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Master's thesis

Production of Polyhydroxybutyrate (PHB) from Desugarized Molasses by *Bacillus megaterium* CECT 7922

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Abstract

Quite recently there has been a growing interest in biopolymers called PHAs (polyhydroxyalkanoates), of which PHB (polyhydroxybutyrate) is the simplest and most common polymer. PHAs, produced by microbial fermentation, have comparable properties to conventional plastics.

This study investigated how to optimize PHB production, using *Bacillus megaterium* CECT 7922, isolated from the Uyuni salt lake, with desugarized molasses as a cheap substrate. The potential use of PHB as food packaging material was equally focused on.

To evaluate the cell dry weight and PHB production, shake-flask cultivation and bioreactor cultivation were carried out, using batch as well as fed-batch processes.

The results show that the highest concentrations of CDW (50.47 gL^{-1}) and PHB (20.21 gL^{-1}) were obtained in fed-batch cultivation whereas batch cultivation showed only half the concentrations. The batch process, however, led to a higher PHB productivity (0.446 $gL^{-1}h^{-1}$) as well as higher yields of CDW and PHB (0.149 and 0.079 g per g desugarized molasses added). The adding of phosphate to the medium improved the biomass production, namely higher biomass concentration and faster growth. Certain media containing KCl concentrations instead of NaCl also enhanced the growth of *Bacillus megaterium* CECT 7922 and its PHB production. It was further revealed that the optimum pH value for PHB production is between 7 and 8 and that PHB production is also feasible using unsterile desugarized molasses, but with less substantial concentrations.

The findings of the literature research indicate that PHB is applicable for food packaging, due to its suitable antimicrobial activity, its gas/water/UV resistance, its thermal stability as well as its tensile strength. The findings of the practical part indicate an improved process of PHB production useable on a larger scale. It is, however, recommended to develop the unsterile fermentation processes further for cost efficient PHB production.

Kurzfassung

In jüngster Zeit ist das Interesse an Biopolymeren, den sogenannten PHAs (Polyhydroxyalkanoate), gewachsen, von denen PHB (Polyhydroxybutyrat) das einfachste und gebräuchlichste Polymer ist. PHAs, die durch mikrobielle Fermentation hergestellt werden, haben vergleichbare Eigenschaften wie herkömmliche Kunststoffe.

Diese Studie untersuchte, wie die PHB-Produktion optimiert werden kann, indem das aus dem Salzsee von Uyuni isolierte *Bacillus megaterium* CECT 7922, mit Restmelasse als billiges Substrat, verwendet wird. Ebenso wurde mittels Literaturrecherche der mögliche Einsatz von PHB als Lebensmittelverpackungsmaterial untersucht.

Zur Beurteilung der Zelltrockenmasse (ZTM) und der PHB-Produktion wurden die Schüttelkolbenkultivierungen und die Bioreaktor-Kultivierungen sowohl im Batch- als auch im Fed-Batch-Verfahren durchgeführt.

Die Ergebnisse zeigen, dass die höchsten Konzentrationen von ZTM (50,47 gL^{-1}) und PHB (20,21 gL^{-1}) in der Fed-Batch-Fermentation erzielt wurden, während die Batch-Fermentation nur die Hälfte der Konzentrationen aufwies. Der Batch-Prozess führte jedoch zu einer höheren PHB-Produktivität (0,446 $gL^{-1}h^{-1}$) sowie zu höheren Ausbeuten von ZTM und PHB (0,149 und 0,079 g pro g Restmelasse). Die Zugabe von Phosphat zum Medium verbesserte die Biomasseproduktion. Mineralmedien, welche KCl statt NaCl enthielten, förderten das Wachstum von *Bacillus megaterium* CECT 7922 und dessen PHB-Produktion. Es zeigte sich weiter, dass der optimale pH-Wert für die Kultivierung zwischen 7 und 8 liegt und dass die PHB-Produktion auch mit unsteriler Restmelasse möglich ist, die Effektivität des Prozesses allerdings geringer ist. Es ist zu empfohlen, die unsterilen Fermentationsverfahren für eine kosteneffiziente PHB-Produktion weiterzuentwickeln.

Die Ergebnisse der Literaturrecherche deuten darauf hin, dass PHB aufgrund seiner geeigneten antimikrobiellen Aktivität, seiner Gas/Wasser/UV-Beständigkeit, seiner thermischen Stabilität sowie seiner Zugfestigkeit für Lebensmittelverpackungen geeignet ist.

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Abbreviations

PHB	polyhydroxybutyrate
PHA or PHAs	polyhydroxyalkanoates
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
3HHx	3-hydroxyhexanoate
3HO	3-hydroxyoctanoate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
4HB	4-hydroxybutyrate
scl-PHA	short-chain length polyhydroxyalkanoates
mcl-PHA	medium-chain length polyhydroxyalkanoates
lcl-PHA	long-chain length polyhydroxyalkanoates
PP	polypropylene
P(3HB-3HV)	poly-3-hydroxybutyrate-3-hydroxyvalerate
HAME	hydroxyalkanoate methyl esters
3HBME	3-hydroxybutyrate methyl esters
HA	hydroxyalkanoic acids
TCA	tricarboxylic acid
CDW (ZTM)	cell dry weight (Zelltrockenmasse)
TKN	Total Kjeldahl Nitrogen
DM	desugarized molasses
B. megaterium	*Bacillus megaterium*

1 Introduction

1.1 Polymers

“We are the children of the plastic age” (Boote, 2009) as stated by Werner Boote in his acclaimed film “Plastic Planet”. Plastics can be found everywhere in daily life; in cosmetics, clothes, medicine, automobiles parts and many more (Koller, 2014; Reddy *et al.*, 2003). Polymers are formed by monomers which consist of one backbone, containing carbon chains with the optional addition of elements such as oxygen, nitrogen or sulfur. Monomers can be repeated several thousand times, which gives a diverse molecular weight to the respective polymer. Beside these repeating units, there are also other molecular groups (side chains) attached to the backbone. Depending on these side chains, polymers have various properties. The conventionally available plastics are mostly derived from petroleum due to lower costs; e.g. polyamide, polycarbonate, polyester, polyethylene, polypropylene, polystyrene, polyurethane and polyvinyl chloride etc.

Polymers have been invented for the first time in the 1860s. Almost 60 years later, they were introduced on an industrial level. The polymer industry has been growing fast in the past decades all over the world, replacing traditionally used materials like glass, metal and paper (Worldwatch Institute, 2015).

Figure 1 shows in which sectors plastics are used in Europe (PlasticsEurope, 2016). The major use of plastics is in packaging, accounting for 40%. 22% of plastics are used in consumer and household goods as well as furniture and sport gears, followed by 20% in building and construction applications. The remaining 18% of plastics usage is in the field of the automotive industry, electronics and agriculture.

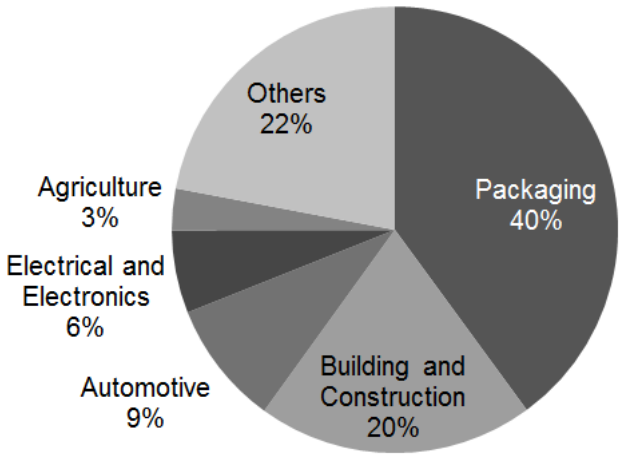


Figure 1: Plastic usage sectors in Europe (PlasticsEurope, 2016).

Plastics are versatile, light, durable and inexpensive (Marina *et al.*, 2017). Plastics have lots of advantages, for example the reduction of food waste by keeping products fresh for longer, improved transportation efficiency (compact boxes wrapped in plastics) and the manufacturing of various devices and building blocks in the industry (less weight) (Worldwatch Institute, 2015).

Considering the disadvantages, the biggest problem is the disposal of plastics. 22 to 43% is discarded in landfills (Kale *et al.,* 2007; Reddy *et al.*, 2003; Worldwatch Institute, 2015). The rest is either dumped in the sea, incinerated or recycled. Each of these methods is partly dissatisfying;

- Landfills: Considering the nearly 300 million tons of plastics produced per year (Worldwatch Institute, 2015) as well as their slow degradation (Baztan *et al*., 2017), the plastic waste takes up considerable space.
- Disposal in the sea: Abandoned plastics in the sea have a negative impact on the environment, as the plastics remain in the oceans. When ingested by fish and sea plants, they get reintroduced into the food chain (Worldwatch Institute, 2015).
- Incineration: In this case, harmful chemicals like bisphenol A, hydrogen chloride and hydrogen cyanide are released during the heat treatment (Johnstone, 1990; Atlas and Bartha, 1993).
- Recycling: The sorting of the various kinds of plastics is difficult (Johnstone, 1990).

The awareness of the environmental damage caused by plastics creates a need to reduce unnecessary plastic consumption and increases the demand for more sustainable materials. Thus, more environmentally friendly alternatives like bioplastics, (biobased and/or biodegradable) have been researched and developed.

Biobased plastics mean polymers derived from renewable sources such as starch, vegetable oil and fat. Biodegradable plastics are materials which can be decomposed by the microorganisms existent in the environment. Reddy *et al.* (2003) classified biodegradable plastics into three types; photodegradable, semi-biodegradable, and completely biodegradable;

- Photodegradable plastics include light sensitive groups bound to the backbone of polymer. The structure of those groups can be opened under extensive ultraviolet radiation, which furthers the bacterial degradation of the polymer. The light is the essential factor to degrade the photodegradable plastics. Thus, they might remain as a non-degraded form in landfills due to the lack of sunlight.

- Semi-biodegradable plastics are starch-linked plastics incorporated into polyethylene. Bacteria, existing in the environment, only degrade the starch thereby leaving the other fragments including polyethylene (a non-degradable plastic).
- Biodegradable plastics (polyhydroxyalkanoates, polylactic acid, aliphatic polyester, polysaccharides and their copolymers) can be produced and completely consumed by microorganisms (Bucci *et al.*, 2007; Gouda *et al.*, 2001).

As this study focusses on polyhydroxyalkanoates, the following chapters will be devoted to the description of their classification, their properties, their applications and their production.

1.2 Polyhydroxyalkanoates (PHAs)

1.2.1 Introduction

PHAs were discovered as a sudanophilic bacterial inclusion at the beginning of the twentieth century. PHAs are intracellularly accumulated by microbial fermentation from various carbon sources, mostly sugar or lipid. They are a form of carbon and energy storage molecules under nutrient limitation conditions (Arrieta *et al.,* 2017; Lu *et al.*, 2009; Reddy *et al.*, 2003; Salgaonkar *et al.*, 2013; Wang *et al.*, 2014). Numerous microorganisms like bacteria, fungi and algae present in the respective environment are able to degrade PHAs (Arrieta *et al.*, 2017).

1.2.2 Chemical structure and classification of PHAs

The PHA is composed of (R)-β-hydroxy fatty acids. The R group varies from methyl (C1) to tridecyl (C13) (Madison and Huisman, 1999).

Typical PHA monomers are 3-hydroxybutyrate (3HB, C4), 3-hydroxyvalerate (3HV, C5), 3-hydroxyhexanoate (3HHx, C6), 3-hydroxyoctanoate (3HO, C8), 3-hydroxydecanoate (3HD, C10) and 3-hydroxydodecanoate (3HDD, C12) as well as 4-hydroxybutyrate (4HB), which can form homopolymers or heteropolymers consisting of at least two different monomers (see Figure 2). On the basis of monomers involving the formation of polymers, they have diverse material properties (Chen *et al.*, 2015; Wang *et al.*, 2014).

Depending on the side chain lengths of monomers, PHAs can be classified into short-chain length and medium-chain length PHAs consisting of 3 to 5 and 6 to 14 carbons, respectively (Gao *et al.*, 2011). PHAs that have more than 14 carbon atoms in the side chain are long-chain length PHAs, but they are uncommon (Raza *et al.*, 2018). Polyhydroxybutyrate (PHB), containing one carbon atom in the side chain, is the most commonly produced polymer among the PHAs. PHB was first identified by Lemoigne in 1926 (Lemoigne, 1926; Sudesh *et al.*, 2000).

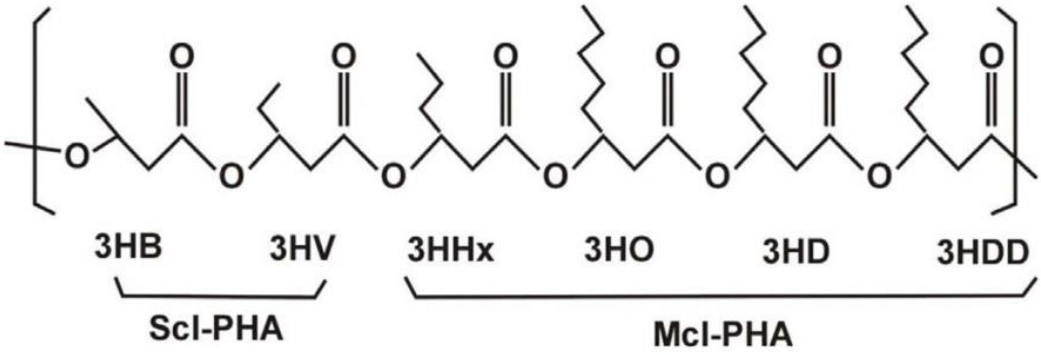


Figure 2: Chemical structure of PHAs (Raza *et al.,* 2018).
Short chain length (scl-PHA) PHA: 3-hydroxybutyrate (3HB); 3-hydroxyvalerate (3HV); Medium-chain length (mcl-PHA): 3-hydroxyhexanoate (3HHx); 3-hydroxyoctanoate; (3HO); 3-hydroxydecanoate (3HD); 3-hydroxydodecanoate (3HDD).

1.2.3 Properties of PHAs

More than 100 different monomers have been identified, which can be combined to produce different types of PHAs as homopolymers or co- and heteropolymers (Raza *et al.,* 2018). These polymers have diverse mechanical and thermal properties. Their biodegradability can also be improved by using biomolecular techniques which result in the change of PHA synthase structure and activity as well as the metabolic pathways (Chen *et al.,* 2015; Sudesh *et al.,* 2000). In general, PHAs have high molecular weight ranging from 50 to 1,000 kDa (Reddy *et al.,* 2003) and are similar to conventional plastics such as polypropylene (Madison and Huisman, 1999). However, PHAs are regarded as eco-friendly green materials, as they are non-toxic, biodegradable and biocompatible (Reddy *et al.,* 2003; Salgaonkar *et al.,* 2013).

Depending on the kind of monomers involved in the formation of polymers, the thermal, mechanical and physical properties of PHAs differ. Table 1 shows these properties of PHAs compared to conventional plastics. To evaluate the mechanical properties, the elongation at break, which measures toughness or total deformation degree before fracture, and the elasticity by Young's modulus as well as the tensile strength measuring the strength before the permanent deformation, should be considered (McChalicher and Srienc, 2007).

Physical properties include molecular weight and crystallinity. Crystallinity can be defined as amorphous (0%) to highly crystalline (>90%). High degree of crystallinity signifies rigidness and high melting point (Balani *et al.,* 2005).

PHB, the simplest and the most common polymer among PHAs, shows high crystallinity, due to simple structural side chains, providing good resistance to moisture and ultraviolet light as well as gas barrier performance (Marina *et al.,* 2017; Raza *et al.,* 2018). The main disadvantage of PHB is that it is brittle with low strain at break. To compensate the high

crystallinity and brittleness of PHB, blending with other polymers like mcl-PHAs can be considered (Marina *et al.,* 2017). Compared to scl-PHAs like PHB or P(3HB-3HV), the mcl-PHAs have a much lower level of crystallinity (Reddy *et al.,* 2003) (not shown in Table 1) and are more elastic.

Regarding thermal properties, PHAs are thermoplastic polymers (Marina *et al.,* 2017). Their glass transition temperature varies from -40 to 4 °C and their melting temperature from 45 to 180 °C (Możejko-Ciesielska and Kiewisz, 2016; Padermshoke *et al.,* 2005) (See Table 1).

PHAs can be degraded by numerous microorganisms present in environments under aerobic or anaerobic conditions. Under aerobic conditions, PHA is degraded to carbon dioxide and water, while anaerobic environments result in carbon dioxide and methane formation (Reddy *et al.,* 2003). PHAs biodegradation is affected by surface area, moisture, temperature, pH level, polymer composition and type of microorganisms (Boopathy, 2000; Masood *et al.,* 2014). When microorganisms are in contact with polymers, they secrete enzymes like extracellular PHB depolymerase to hydrolyze PHB into monomers and/or oligomers (Bucci *et al.,* 2005; Sudesh *et al.,* 2000).

Table 1: Comparison of PHA polymers with conventional plastics regarding thermal, mechanical and physical properties.

(Reference: 1. Doi *et al.,* 1990; 2. Możejko-Ciesielska and Kiewisz, 2016; 3. Saito and Doi, 1994)

		Thermal properties		**Mechanical properties**			**Physical properties**		**Refer-ence**
		Melting temperature (°C)	Glass transition temperature (°C)	Elongation at break (%)	Young's modulus (GPa)	Tensile strength (MPa)	Molar weight (10^5 g/mol)	Crystallinity (%)	
PHAs	Homopolymer, scl-PHAs (P3HB)	180	4	3.5	40	5	1 to 8	80	1, 2
	Homopolymer, mcl-PHAs	45 to 80	-40	-	300	20	-	-	2, 3
	Copolymer, P(3HB-co-3HV)	83 to 170	-13 to 10	0.7 to 2.9	30 to 38	up to 690	3	55 to 70	1, 2
	Copolymer, P(3HB-co-3HD)	130	-8	-	680	17	-	-	2
Conven-tional plastic	Polypropylene	176	-10	1.7	38	400	2.2 to 7	70	1, 2

1.2.4 Applications

PHAs can be utilized in many fields, due to their diverse properties (thermoplasticity, biodegradability and non-toxicity) as mentioned in the above chapter (Możejko-Ciesielska and Kiewisz, 2016).

They were firstly used for cosmetic packaging materials (Hocking and Marchessault, 1994) and later also for food and other daily consumable goods due to their good gas and vapor resistance (Marina *et al.*, 2017; Reddy *et al.*, 2003; Salgaonkar *et al.*, 2013).

PHAs can also be transformed into hydroxyalkanoate methyl esters (HAME) under acidic conditions, which can be used as a fuel additive (Raza *et al.,* 2018; Wang *et al.,* 2010; Zhang *et al.,* 2009). The fuels blended with HAME, especially, 3HBME was reported to have better properties compared to ethanol, in terms of oxygen content, dynamic viscosity and boiling point (Wang *et al.,* 2010). Moreover, Bond-Watts *et al.* (2011) reported that biofuel butanol can be produced from glucose through the combination of PHA synthesis and other metabolic pathways.

Furthermore, PHAs are non-toxic and have a low immunogenicity. PHAs can thus be used for drug delivery systems, acting as an outer shell. When the PHA coating has been degraded, the drug is released (Raza, Abid, and Banat, 2018). A degraded form of PHB, D-3-hydroxybutyrate (HB), can be found in the human blood as a natural biocompatible constituent (Khosravi-Darani, 2015). For this reason the medical applications of PHAs have been investigated, especially PHB (Kulpreecha *et al.,* 2009). It is for example applicable to osteosynthetic materials like bone plates, surgical sutures and blood vessel replacements. It stimulates bone growth because of its piezoelectric properties (Reddy, Ghai, and Kalia, 2003; Salgaonkar, Mani, and Braganca, 2013). Moreover, Zou *et al.* (2009) showed that 3HB has a potential medical application related to Ca-channel activation and memory enhancement.

Another possible application is using PHA monomers, which are mostly chiral hydroxyalkanoic acids (HA). They can be used as precursors or intermediates for the synthesis of compounds like antibiotics, food additives, aromatic substances and vitamins (Chen and Wu, 2005; Ren *et al.,* 2010). Additionally, PHAs are considered as raw materials for the production of dyes (Redd *et al.,* 2003) and as carriers for long term release of herbicides of insecticides in agriculture (Galego *et al.,* 2000; Kulpreecha *et al.,* 2009). The ultrahigh molecular weight of PHAs allows to produce strong fibers for the fishery industry (Bugnicourt *et al.,* 2014).

1.3 Production of PHAs

1.3.1 Metabolism

PHA formation by microorganisms mostly occurs under stress conditions such as nitrogen, phosphorus or oxygen limitation. The limitation conditions trigger a metabolic pathway which results in PHA production. PHA as a form of carbon reserve material allows the microorganisms to survive in challenging environments (Chinwetkitvanich *et al.,* 2004; Du *et al.,* 2001).

More specifically, the nutrients limitation leads to an overproduction of acetyl-CoA (Guevara-Martínez *et al.,* 2015). In addition, it was found that enzymatic activities of β-ketothiolase and acetoacetyl-CoA reductase, which are involved in PHA biosynthesis, increased when nutrients were limited (Ryu *et al.,* 1997). The increased activity of the above two enzymes ensures the availability of acetyl-CoA to enter the PHA biosynthetic pathway. In contrast, β-ketothiolase is negatively regulated under non-limited conditions (Du *et al.,* 2001; Kessler and Witholt, 2001).

The main PHA biosynthetic pathway consists of three steps (Kessler and Witholt, 2001; Reddy *et al.,* 2003; Tsuge, 2002).

- Firstly, two acetyl-CoA molecules convert to acetoacetyl-CoA by β-ketothiolase (encoded by PhaA).
- Secondly, acetoacetyl-CoA is reduced to (R)-3-hydroxybutyrl-CoA in a catalyzed reaction by NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by PhaB).
- Lastly, (R)-3-hydroxybutyrl-CoA is polymerized by PHB synthase (encoded by PhaC), resulting in PHA.

There are further pathways called the methylmalonyl-CoA pathway and the de novo fatty acid synthetic pathway (Gao *et al.,* 2011; Reusch, 2013; Sudesh *et al.,* 2000; Tsuge, 2002) (see Figure 3).

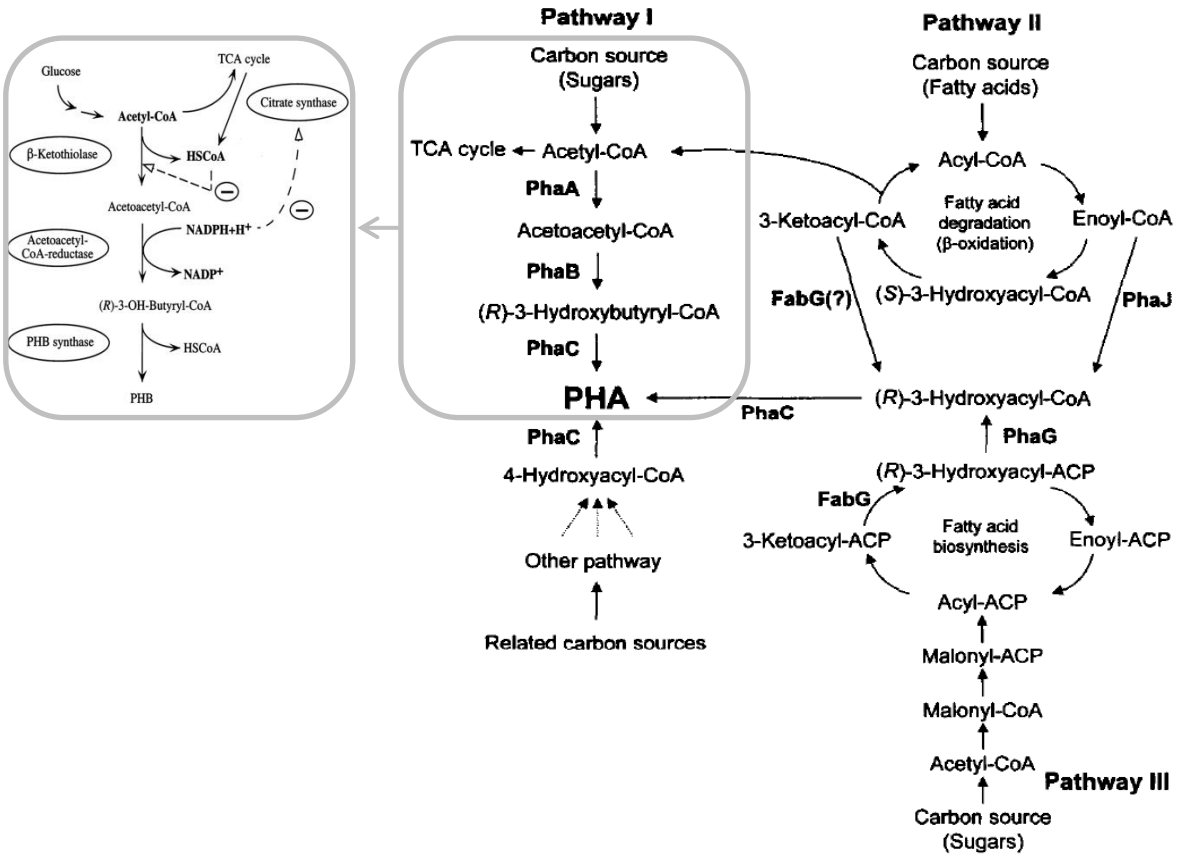


Figure 3: Metabolic pathways for PHA biosynthesis (Modified figure from Kessler and Witholt, 2001; Tsuge, 2002).

1.3.2 Microbial fermentation

There are more than 90 genera of both gram-positive and gram-negative bacteria producing PHAs under aerobic or anaerobic conditions (Kim *et al.*, 2007; Zinn *et al.*, 2001). Bacteria accumulate PHAs in the cytoplasm as granules (0.2 – 0.5 µm) (Raza *et al.*, 2018) mostly under nutrient limiting conditions. However, it is also seen that some organisms are able to produce PHA during growth phase without any nutrient limitation (Muhammadi *et al.*, 2015). Bacteria such as *Cupriavidus necator* (*Ralstonia eutropha)*, *Pseudomonas oleovorans* and *Pseudomonas putida* accumulate PHAs under nutrient limitation conditions, while there are some microorganisms like recombinant *Escherichia coli* that can produce PHAs during the growth phase without requirements of nutrient limitation (Nitschke *et al.*, 2011).

1.3.2.1 PHA producing microorganisms

PHA producing gram-positive bacteria

Gram-positive bacteria such as *Bacillus*, *Clostridium*, *Corynebacterium*, *Rhodococcus*, *Streptomyces* and *Staphylococcus*, have been in place as PHA producers for a long time due to their high reproducibility (Macrae and Wilkison, 1958; Valappil *et al.*, 2007a). Sporulation of gram-positive bacteria is triggered by similar growth situations, and is therefore competitive to PHA formation (Wu *et al.,* 2001; Chen, 2010). Unlike gram-negative bacteria, PHA produced by gram-positive bacteria does not contain lipopolysaccharides (LPS) (Valappil *et al.*, 2007a). This is beneficial as the presence of LPS is not desirable for the medical applications due to possible immunogenic reactions.

A number of research studies on gram-positive bacteria have shown that they can produce homopolymers, namely PHB and PHV, and its co-polymers (Raza *et al.*, 2018; Valappil *et al.*, 2007a).

PHA producing Bacillus species

Bacillus species are among the first gram-positive bacteria producing PHA, which have been investigated on a scientific level. A reason for this might be that the *Bacillus* spp. do not produce lipopolysaccharide and they secrete a number of enzymes capable to metabolize various substrates (Halami, 2008). It was proven that *Bacillus* spp. can use simple carbon sources like sugar or complex carbon sources from waste products to produce PHAs (Bhuwal *et al.*, 2013; Singh *et al.*, 2009; Sonakya *et al.*, 2001). *Bacillus* spp. are used not only for PHA formation, but also for the production of different compounds such as surfactants, antibiotics and flavor enhancers etc. (Salgaonkar *et al.*, 2013).

Table 2 presents *Bacillus* spp. identified as PHA producers. The PHB content obtained by *Bacillus* spp. varies from 20 to 65% (w/w of CDW – up to 70 g/L), which indicates a reasonable PHB production (Chaijamrus and Udpuay, 2008; Gouda *et al.*, 2001; Halami, 2008; Kumar *et al.*, 2009; Omar *et al.*, 2001; Rodríguez-Contreras *et al.*, 2013; Rodríguez-Contreras *et al.*, 2016; Salgaonkar *et al.*, 2013; Wu *et al.*, 2001).

Table 2: PHA producing *Bacillus* species.

Microorganism	Carbon source	PHA	PHA content (%w/w)	Biomass concentration (g/L)	References
Bacillus megaterium	Sugarcane molasses	PHB	44	1.4	Gouda *et al.*, 2001
***Bacillus megaterium* QMB1551**	Glucose	PHB	20	-	Floccari *et al.*, 1995
Bacillus megaterium	Date syrup Beet molasses	PHB	52 50	3.3 3.7	Omar *et al*., 2001
***Bacillus megaterium* uyuni S29**	Glucose +45% NaCl	PHB	41	3.3	Rodríguez-Contreras *et al.*, 2016
***Bacillus megaterium* uyuni S29**	Glucose +0.9 g/L NaCl	PHB	30	28.6	Rodríguez-Contreras *et al*., 2013
***Bacillus megaterium* ATCC 6748**	Sugarcane molasses Corn steep liquor	PHB	35 40	3.5 6	Chaijamrus and Udpuay, 2008
***Bacillus megaterium* H16**	Glucose	PHA	40	-	Salgaonkar *et al.*, 2013
***Bacillus cereus* CFR06**	Glucose Sucrose Starch	PHA	50 46 48	1.4 1.5 1	Halami, 2008
***Bacillus cereus* EGU44**	Biowaste (pea-shell slurry) Glucose Sucrose	PHB	65 50 35	0.95 0.44 0.17	Kumar *et al*., 2009
***Bacillus* sp. JMa5**	Molasses	PHB	30	70	Wu *et al.*, 2001

Bacillus megaterium as a PHB producer

Bacillus megaterium strains can accumulate PHB of 20-41% (of CDW) using glucose as carbon source (Rodríguez-Contreras *et al.*, 2013; Rodríguez-Contreras *et al.*, 2016; Floccari *et al.*, 1995; Salgaonkar *et al.*, 2013). An even higher percentage of PHB (35-52% of CDW) is achieved, when renewable carbon sources such as sugarcane molasses, beet molasses and date syrup is being used (Chaijamrus and Udpuay, 2008; Gouda *et al.*, 2001; Omar *et al.*, 2001). As desugarized sugar beet molasses is to be used in this study *Bacillus megaterium* uyuni S29 was chosen.

Bacillus megaterium uyuni S29 (CECT 7922), isolated from the Bolivian salt lake Uyuni (Rodríguez-Contreras *et al.,* 2013; Rodríguez-Contreras *et al.,* 2016) was purchased at the "Spanish Type Culture Collection (CECT)"

Uyuni salt lake has a neutral pH (around 7.4) and a salt brine layer of 10-20 cm under the surface crust (Risaçher and Fritz, 2000). The ions present in Uyuni salt lake are shown in Table 3 along with the data from other saline environments such as deep seas and salt lakes. All show similar concentrations of Ca^{2+}, Mg^{2+} and SO_4^{2-}. The Uyuni salt lake, however, has a relatively higher Na^{+} concentration and much higher concentration of K^{+}.

The experiments conducted in the wake of this study revealed that it is a rod-like, gram-positive and non-spore forming bacterium.

Table 3: Concentration of cations and anions in various hypersaline brines and seawater.

(Reference: 0. Perez-Fernandez *et al*., 2016; 1. Javor, 1989; 2. Grant, 2004; 3. Wielen *et al*., 2005)

	Na^+ (g/L)	K^+ (g/L)	Ca^{2+} (g/L)	Mg^{2+} (g/L)	Cl^- (g/L)	SO_4^{2-} (g/L)	Salinity	Reference
Uyuni salt flat	93.9	15.0	0.48	15.0	192.8	17.1	-	0
Seawater	10.8	0.4	0.4	1.3	19.4	2.7	35	1
Great Salt lake (North Arm), USA	105.0	6.7	0.3	11.1	181.0	27.0	333	1
Dead Sea, Israel-Jordan	39.7	0.2	17.2	42.4	219.0	0.4	327	1
Don Jan Pond, Antarctica	11.5	2.3	114.0	1.2	212.0	0.01	339	1
Lake Magadi, Kenya	161.0	2.3	0	0	111.8	16.8	315	2
Bannok deep-sea brine lake, Mediterranean	97.4	5.0	0.7	15.8	190.0	13.2	322	3
Urania deep-sea brine lake, Mediterranean	80.6	4.8	1.3	7.7	132.2	10.3	237	3
Discovery deep-sea brine lake, Mediterranea	1.6	0.8	0.1	121.4	336.5	9.2	470	3

1.3.2.2 Limitation methods

The common strategy of PHB production is a two-stage approach. Firstly, the cells are given relatively rich nutrients to reach the maximum growth, and then one or two nutrients are limited in order to trigger the PHA accumulation (Chinwetkitvanich *et al.*, 2004; Du *et al.*, 2001).

Nitrogen limitation

Most PHAs producing bacteria accumulate PHAs under nitrogen limitation (Brandl *et al.,* 1990; Omar *et al.*, 2001). Thus, many studies have investigated the effect of nitrogen limitation (indicated by C/N ratio) on cell growth and PHA accumulation. It was revealed that an increase of C/N ratio leads to better PHA accumulation (Ahn *et al.*, 2015; Cui *et al.*, 2017). For example, the PHA accumulation by *Haloferax mediterranei* was 47.22% of CDW at a C/N ratio of 35 (Cui *et al.*, 2017). An increased C/N ratio of 129 resulted in a PHA accumulation of 59% of CDW (Wen *et al.*, 2010).

Phosphorus limitation

Phosphorus limitation is another way to induce the cells to produce PHB. Ryu *et al.* (1997) reported that PHB content using *Cupriavidus necator* (*Alcaligenes eutrophus)* under phosphate limitation reached 80% of CDW. A significant PHB accumulation started when the phosphate concentration was below 0.4 g/L. It was remarked that sufficient phosphate should be supplied at the beginning of the fermentation to increase the cell growth, as the increase of the cell growth is related to the increase of PHB production later on.

Oxygen limitation

Pandian *et al.* (2010) found that PHB accumulation was induced at a low level of dissolved oxygen. Significant PHB accumulation was shown at 60% of dissolved oxygen and its maximum was reached when dissolved oxygen remained at 10%. Oxygen limitation might be an efficient way to establish the harsh environment as less energy is required. Moreover it benefits large scale fermentation, as the supply of oxygen is challenging (Wei *et al.,* 2009).

1.3.2.3 Carbon sources

During both stages, namely the growth and nutrients limiting phases, carbon sources should be sufficient. Better results can be achieved through the fed-batch fermentation resulting in maximal bacterial growth and PHB production. As carbon source is one of the main reasons for high production cost, a lot of studies investigate PHA production with alternative carbon (Nonato*et al.,* 2001). Research has found rice, starch, plant oils and agricultural waste to be useful for this purpose (Getachew and Woldesenbet, 2016; Haas *et al.*, 2008; Huang *et al.*, 2006; Raza *et al.*, 2018; Tsuge, 2002).

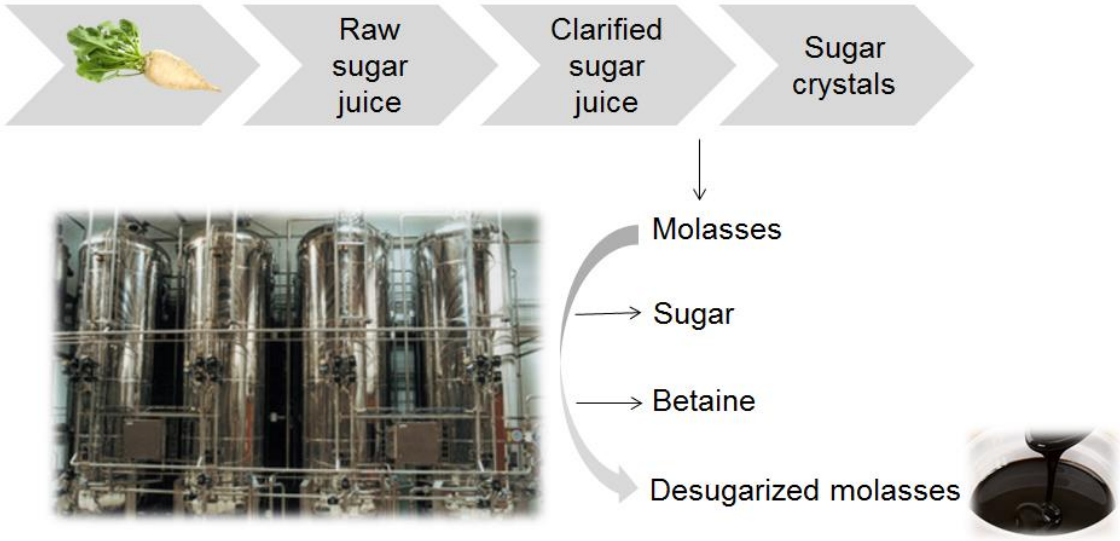
Rice or starch can be used as a cheap renewable resource as they are cultivated worldwide. For example, high biomass and PHA production were obtained from the mixture of rice bran and starch, 140 g/L and 77.8 g/L, respectively (Huang *et al.*, 2006). When potato starch as a carbon source was used, 179 g/L of biomass with 55% PHA accumulation in the cells was achieved (Haas *et al.*, 2008).

Used plant oils are also an inexpensive carbon source, as they can be supplied by fast food companies as a form of waste disposal (Raza *et al.*, 2018). Plant oils consist to a large part of fatty acids. This is an advantage for the production of PHA, as yields from fatty acids substrates are considerably higher than those based on glucose. It needs to be mentioned however that microorganisms show a slower growth rate when using fatty acids (Tsuge, 2002).

Furthermore, agricultural waste, such as whey from the cheese and casein industry as well as molasses from the sugar industry, is also a cheap raw material. As the waste is used as a substrate for the fermentation, not only the PHA production costs, but also the impact on the environment caused by the disposal of this waste can be reduced (Getachew and Woldesenbet, 2016).

Molasses is a byproduct from the sugar manufacturing or soy processing industry. It is being used as a substrate for PHB production, since the molasses contains mainly sucrose and other carbohydrates that can be used as carbon sources for microorganisms (Shasaltaneh *et al.*, 2013; Purushothaman *et al.*, 2001). Some studies showed even better growth and PHB accumulation, when the molasses was applied instead of simple sugar. This might be explained by trace elements or other minor components present in molasses (Gouda *et al.*, 2001; Omar *et al.*, 2001; Purushothaman *et al.*, 2001).

In this study, desugarized molasses was used as a substrate. A local manufacturing company (AGRANA), using sugar beets as raw material, served as the supplier. Desugarized molasses, sometimes also referred to as “desugarized molasses” differs compared to “normal” molasses. Usually the sugar production can be separated in the following steps: sugar beet is turned into sugar juice, then clarified and crystalized, leaving the molasses as a by-product. To gain the desugarized molasses, the “custom” molasses undergoes an additional chromatographically extraction step. As a result, three extraction phases are produced. The first phase is the sugar phase, the second phase extracts the betaine and the third phase is the raffinate. By evaporating the water, the raffinate concentrated, resulting in the so called desugarized molasses. Picture 1 shows an overview of the desugarized molasses production.



Picture 1: Desugarized molasses derived from sugar beet (Novasep, 2018; OrganicFacts, 2017; Ufuk Tarım, 2018)

1.3.3 Downstream

Depending on the extraction and purification strategies, the production costs can be different (Scheller and Conrad, 2005). The recovery rate, showing the amount of PHAs extracted from the cell, is a critical point for efficient extraction. The level of purity might differ depending on the application. For example, for medical purposes purity is more important than the total cost. For PHAs packaging materials (especially disposable items) however, the total cost is more important than high purity (Raza *et al*., 2018).

To extract PHAs, many methods have been developed. Among them, solvent extraction is the most common way due to its easy handling (Raza *et al.*, 2018). PHAs are insoluble in water but they are soluble in chlorinated solvents. Consequently various chlorinated solvents such as chloroform, dichloromethane and sodium hypochlorite are being used (Gamal *et al.*, 2013; Padermshoke *et al*., 2005). The recovery rate can be improved by rupturing the cells, as PHAs are released from the cells, leading to a better solubility in the solvent (Raza *et al.*, 2018). One of the methods to rupture the cells is using enzymatic reactions (Jacquel *et al.*, 2008).

As the chlorinated solvents are toxic, some environmentally friendly downstream processes without these solvents have also been reported such as super critical fluids extraction, aqueous two-phase extraction and mechanical treatment (Raza *et al.*, 2018). The purity and recovery level are not comparable to chlorinated solvents, making further improvements necessary (Ghatnekar *et al.*, 2002; Ramsay *et al.*, 1990).

1.3.4 Industrial production of PHAs

PHA producing companies can be found all over the world, but the scale of their production is still smaller compared to other biopolymers and conventional plastics. In recent years a rapid growth could be observed, as the interest for PHAs is on the rise (Laura, 2017). More detailed information on this topic is provided in chapter 5.3 Market and Table 21.

1.3.5 Economic aspects

PHAs are priced depending on many factors, namely, the substrate cost, PHA yield (g PHA per g substrate) and the downstream process (Serafim *et al.*, 2004). The cost composition of PHB production has been estimated by Nonato *et al.* (2001) as follows. Substrate, in terms of raw materials and other chemicals, accounts for almost half the cost. Equipment depreciation occupies one third of the total cost and 11% go to energy usage. Based on this estimation, it is clear that the reduction of the substrate cost by using renewable resources or waste streams can decrease the overall costs of PHB substantially. The costs of the downstream process on the other hand can be lowered by a higher PHA recovery and purification level (Wang *et al.*, 2014).

2 Objectives

The aim of this study is the process optimization of PHB production by *Bacillus megaterium* uyuni S29 (CECT 7922) using desugarized molasses as a substrate. Subsequently this optimized process can then be applied for upscaling purposes.

For this purpose, various process parameters, such as media compositions, fermentation conditions and feeding strategies will be investigated to improve the process toward a better biomass as well as PHB production. Furthermore, the influence of these parameters on the yield of PHB and the potential properties will be examined.

First, the substrate from different batches will be inspected to investigate if different desugarized molasses batches influence the growth behavior and PHB formation. Subsequently it will be examined if the adding of salt, betaine, iron or phosphate to the substrate can increase the content of PHB.

Then, the effect of pH values on PHB production will be evaluated. Additionally, an unsterile condition as well as different heat treatments of desugarized molasses such as pasteurization and sterilization will be tested.

As a further step, fed-batch fermentation will be carried out, using a feed shot to maximize the biomass content, and consequently PHB accumulation. Afterwards, the yield and productivity of the batch cultivation and the fed-batch cultivation will be compared.

Lastly, the potential properties of PHB as a packaging material will be investigated by means of literature research.

3 Materials and Methods

3.1 *Bacillus megaterium* uyuni S29 as a PHB producer

Bacillus megaterium strain uyuni S29 (CECT 7922) was stored on agar plates at 4 °C. 15 or 20% (w/w) desugarized molasses (Table 10) agar plates were used. The strain was transferred by an inoculation loop to a fresh agar plate and was cultivated at 35 °C for three days before the pre-culture inoculation. For long term preservation it was stored in a cryo-culture at -80 °C in BX media with 10% glycerol.

3.2 Media

The BX medium

The composition of the BX medium used for the pre-culture is shown in Table 4.

Table 4: Composition of the BX medium.

Chemical	Concentration (g L^{-1})
Meat extract	10
Peptone from casein	10
NaCl	5

The synthetic media

Two synthetic media were used for the production of PHB under controlled nutrient condition. The synthetic media I and II were divided into separate groups (Table 5), sterilized at 121 °C for 20 min and mixed together afterward. Medium II contained either NaCl or KCl in different concentrations according to the experimental plan. The salt solutions were prepared in stock (Table 6) and diluted to the requested concentration. The maximum molarity was 1.71M. The lower concentrations (0.086M, 0.17M, 0.43M, 0.86M, 1.28M) were achieved by diluted the stock solution with reverse osmosis water.

The pH value of the media was adjusted to 7 before the inoculation, using base and acid solutions (2M NaOH and 1M H_2SO_4, respectively).

Table 5: Composition of the synthetic medium I and II.

	Chemical	Synthetic medium I	Synthetic medium II
Group 1	$CaCl_2$ (g L^{-1})	0.02	0.02
	Citric acid (g L^{-1})	0.75	0.75
	KH_2PO_4 (g L^{-1})	2	3.7
	$K_2HPO_4 \cdot 3H_2O$ (g L^{-1})	-	7.5
	Na_2HPO_4 (g L^{-1})	0.6	0.6
	5xSL6 trace elements (mL L^{-1})	1	1
	NaCl (g L^{-1})	10	-
Group 2	Sucrose (g L^{-1})	40	40
	$MgSO4 \cdot 7H_2O$ (g L^{-1})	3	3
Group 3	$FeSO_4$ (g L^{-1})	0.025	0.025
Group 4	$(NH_4)_2SO_4$ (g L^{-1})	2	2

Table 6: The salts used for the synthetic medium II.

	NaCl	KCl
Group 5	100 g L^{-1} / 1.71M	127.65 g L^{-1} / 1.71M

The desugarized molasses

The desugarized molasses obtained from the sugar manufacturing company AGRANA was used as a substrate. Two batches containing desugarized molasses, one from 2016 and another one from 2017, were provided by the company. The chemical composition of desugarized molasses and its pH value are shown in Table 7.

The desugarized molasses was diluted with reverse osmosis water (w/w) to obtain the concentrations, as stated in Table 8. Desugarized molasses with a 20% (w/w) concentration was used for pre-culture. Depending on the experiment conducted, desugarized molasses concentrations of 15 to 40% (w/w) served as media. Less diluted desugarized molasses (60, 80 and 100% (w/w)) was used for feeding. After the preparation the media were sterilized at 121 °C for 20 min. For the experiments, IFA Nr.51, 63, 70 and 94, unsterilized desugarized molasses was used.

The media used for experiments IFA Nr.42, 57 and 65 contained certain amounts of betaine, iron and phosphate, as listed in Table 9. Betaine molasses (molasses collected before the betaine extraction), provided by AGRANA was used as betaine source. 1% (w/w) of betaine molasses, equivalent to 4.3 g/L betaine, was mixed to the 15% (w/w) desugarized molasses.

It has to be mentioned that for the most part desugarized molasses collected in 2017 was used. For experiment IFA Nr.52, the 15% desugarized molasses was prepared two times; once with the desugarized molasses from 2016 and the other time with the one from 2017.

Table 7: The chemical composition and pH value of desugarized molasses (Agrana 2016&2017).

	Batch (2016.05)	Batch (2017.08)
Sucrose	16.94%	13.00%
Lactic acid	6.82%	3.96%
Nitrogen	2.07%	1.78%
Phosphorous	<0.02%	0.03%
pH	6.52	7.48

Table 8: Media composition for each experiment; IFA Nr. means the experimental number assigned in the fermentation working group at IFA Tulln.

IFA Nr.	Media	
	Batch	Feed
Pre-culture		
-	20%	
4.1 Media conditions		
52	15%	-
47	17%, 20%, 25%, 30%, 35%, 40%	-
42	15%	-
	15%+Betaine	
	15%+Iron	
	15%+Phosphate	
4.2 Fermentation conditions		
48	20%	
51	20% (unsterile)	
63	20%	
	20% (unsterile)	
4.3 Feeding strategies		
54	20%	60%, 80%
57	20%	100%
	20%+P	100%
65	20%	80%
	20%+P	80%
70	20%	80% (unsterile)
94	20%	60% (unsterile)
	20%	80% (unsterile)

Table 9: Composition of additional components to the batch media.

	IFA Nr. 42	IFA Nr. 57	IFA Nr. 65
Betaine (g L^{-1})	4.3	-	-
Iron, Fe^{2+} (g L^{-1})	0.009	-	-
Phosphate, PO_4^{3-} (g L^{-1})	0.133	0.133	0.070

The desugarized molasses agar plates

For cultivation of *Bacillus megaterium*, agar plates consisting of desugarized molasses (concentration 15 or 20%, w/w) and 15 g/g of agar were used (Table 10).

Table 10: Composition for 15 and 20% of desugarized molasses agar plates.

	15% DM agar plate	20% DM agar plate
Desugarized molasses (DM) (g)	150	200
Reverse osmosis water (g)	850	800
Agar (g)	15	15

3.3 Fermentation Procedures

3.3.1 Pre-culture

Desugarized molasses agar plate was inoculated with *Bacillus megaterium* CECT 7922 by transferring an active culture with an inoculation loop on the agar plate. It was incubated for approximately 72 hours at 30 °C (HERAEUS incubator, Thermo scientific). 300 mL shaking flasks containing 100 mL of BX media or desugarized molasses (15 or 20% (w/w)) were inoculated by transferring a loopful of colonies from the plate. When desugarized molasses was used the pH was set to 7 with 1M H_2SO_4. The pre-culture was cultivated for 8-24 hours at 35 °C with 130 rpm (INFORS HT Multitron Pro).

3.3.2 Cultivation in shake-flasks

Shake-flask cultivation was carried out in 300 mL Erlenmeyer flasks containing 100 mL of diluted desugarized molasses or the synthetic medium II. The pH value of the media was adjusted to 7 using base/acid solutions (2M NaOH and 1M H_2SO_4). 100 mL of sterilized medium was inoculated with 1 mL of pre-culture and incubated in the shaker (INFORS HT Multitron Pro) at 35 °C with 130 rpm for up to 168 hours. Samples were taken for further analysis every 2 to 4 hours, depending on the growth behavior. Glanapon was used as antifoam to inhibit foam formation.

Table 12 shows an overview of shake-flask cultivation experiments performed in this study.

Picture 2: Shake-flask cultivation in the shaker.

3.3.3 Cultivation in a parallel bioreactor system

Setup

Batch and fed-batch experiments were executed in a parallel bioreactor system (DASGIP, Germany) consisting of 4 glass bioreactors (each 1.4 L) with impellers. The working volume is 800 mL. The DASGIP system used in these experiments was assembled with Bioblock, pH probes with 12 x 225 mm (Mettler Toledo, 405-DPAS-SC-K8S/225), pO_2 probes with 12 x 120 mm (Hamilton, VisiFerm DO ECS 225 H0 or Mettler Toledo, InPro 6800 Series), a pH and pO_2 control module (PH4PO4), a temperature and stirrer control module (TC4SC4), a pump module (MP8), a rotameter gassing station and a cooling machine (Huber, Unichiller). The control software v4.0 was used to regulate the process parameters.

Preparation of the solutions

As solutions were prepared:

- pH calibration solutions with 3 mL in 15 mL falcon tube (four times with pH 7.0 and four times with pH 4.0)
- solutions for CIP procedure (Clean-in Place), namely 70% ethanol, 2.5M NaOH and sterile reverse osmosis water
- acid/base solutions (5M H_2SO_4 and 5M NaOH)
- glanapon (antifoam)

Preparation of the system and the bioreactors

To start the fermentation following actions were performed:

- Set up of the control system

  The control software v4.0 was started and used to set up the process parameters for each experiment. First the four bioreactors to be used were selected. Then pH calibration, oxygen calibration, pump calibration, CIP and cleaning steps were added. For the fermentation conditions, the temperature and the pH value were set to 35 °C and at 7.0 (except for the experiment with the different pH values). The dissolved oxygen level was set at 20% air saturation with a gas flow 0.5-1.0 vvm (L/min).
- pH probes calibration

  Before usage the refrigerated pH calibration solutions were heated up to reached room temperature. Simultaneously the pH probes, which had been stored in 3M KCl, were washed with reverse osmosis water and immersed into a solution with pH 7.0. After connecting the probes to the cables, the “Default” button was clicked in order to prevent old data from interfering with the calibration. Once the values were stable, the “Calibrate Offset” button was pressed. When the symbols had changed from yellow to green, the pH probes again were washed with reverse osmosis water and then placed

in the solution with pH 4.0. After values had stabilized, the “Calibrate Slope” button was pressed. Once the symbols had changed from yellow to green, the pH probes were ready for placing in bioreactors. No change of color in the symbols signified that the probes had not been calibrated. As a result, the steps mentioned above had to be repeated.

- Preparation of oxygen probes

  Either a polarographic or an optical oxygen probe was used.

  The polarographic probe had to be inspected for potential holes in the membrane. If present, the membrane needed replacement. Consequently, the probe was filled with O_2 electrolyte.

  The optical oxygen probe had to be inspected regarding its sensing membrane. If needed, it was cleaned using an isopropanol and a soft cloth.

- Assembling of the bioreactors

  First the air line had to be positioned under the impeller without touching the bottom of the bioreactor. In a next step the calibrated pH probes as well as the prepared oxygen probes were placed in bioreactors. The sockets were covered with either tin foil or a cap. To ensure sterile air inlet, 0.2 µm air filters were connected to the air inlet and covered with tin foil for sterilization. The tubes used for the base trap were attached to the air outlet and clamped together with riser clamps. The luer-lock tube used for sampling was also clamped.

  After the assembling the media was added to the bioreactor. A working volume of 800 mL was applied. The bioreactors and the media were autoclaved at 121 °C for 20 minutes.

- Pump calibration

  For pump calibration 8 falcon tubes (each 15 mL) were covered with parafilm and the tare weight was determined. The tare values were entered to the control system. The acid/base tubes were connected to the corresponding falcon tubes. After 1 hour of dispensing, the tubes were weighed in again. The values were entered again in the system and the calibration was performed automatically.

- CIP procedure (Clean-in-place)

  The CIP procedure (Clean-in-place) was executed with 70% ethanol, 2.5M NaOH, sterile reverse osmosis water and the acid/base solutions (5M H_2SO_4 and 5M NaOH). The first three solutions were run for half an hour each, while the tubes were rinsed with the acid/base solutions for 15 minutes at the end.

- Placing the bioreactors

  The autoclaved bioreactors were placed in the bioblock. All the screws and the lids were tightened and the tin foil was removed. Then, all the tubes (air, acid/base feed

solutions, condenser) and the pH and the oxygen cables as well as the motor and the thermometer were connected.

- Oxygen probes calibration

  Before calibrating the oxygen probes, the temperature was set at 35 °C and the cooling water for the condenser as well as the agitation were turned on to avoid the evaporation of the medium. A two-point calibration was conducted. Old calibration data was deleted by clicking the "Default" button. For maximum O_2-concentration the stirrer speed was set to maximum (1600 rpm) and compressed air was used as oxygen source. The airflow was switched on and as the signal of the O_2 electrode was stable the "Calibrate Slope" button was pressed. After the symbols had turned from yellow to green, the air was switched to N_2. The "Calibrate Offset" button was pressed once the values had stabilized. After calibration, the air was switched back to O_2. The oxygen control setting was set to a p-value of 0.2 and a Ti-value of 150.

Fermentation

After system and the bioreactor preparation, the inoculation was conducted with 50 mL of pre-culture through the luer-lock tube. If fed-batch was conducted, different amounts of substrate were added through the luer-lock tube according to the experimental plan. The concentration of the feed varied from 60 to 100% (w/w) of desugarized molasses and it was either sterile or unsterile. Table 12 shows an overview of bioreactor cultivation (DASGIP) experiments performed in this study.

Samples were taken regularly after inoculation. As the sugar is unequally distributed in the desugarized molasses, a sample was taken before and after the feed shot was added to get more accurate results.

The temperature, the pH value, the dissolved oxygen and the agitation speed were monitored by the DASGIP control system. The pH value of the medium was controlled at 7.0. (except for the experiment with the different pH values). The dissolved oxygen level was automatically controlled by increasing or decreasing the agitation speed 400 to 1200 rpm during the fermentation period.

After the fermentation

Once the fermentation was finished, the bioreactors were disassembled. All the cables, tubes and motor were disconnected from the bioreactors. Afterwards, the tubes used for acid/base solutions were cleaned with sterile reverse osmosis water for half an hour. The oxygen air flow was closed and the cooling machine was turned off. The data was automatically exported to the respective folder.

The procedure of DASGIP system is shown in Table 11 and Picture 3 shows the cultivation with this system.

Table 11: The procedure of DASGIP system.

Preparation of the solutions	
Preparation of the system and the bioreactors	Set up of the control system pH probes calibration Preparation of oxygen probes Assembling of the bioreactors Pump calibration ClP procedure Placing the bioreactors Oxygen probes calibration
Fermentation	
After the fermentation	Disassembling of the bioreactors Cleaning the tubes of acid/base solutions Exporting the data

Picture 3: Cultivation with the DASGIP system.

Table 12: Overview of experiments carried out in this study; IFA Nr. means the experimental number assigned in the fermentation working group at IFA Tulln.

IFA Nr.	Type of fermentation		Experiment conditions	Nutrient limitation method
4.1 Media conditions				
52	Shake flask	Batch	Substrate from different batches	P
47	Shake flask	Batch	Different concentrations of substrate	P
39	DASGIP	Batch	Synthetic medium I	N
91	Shake flask	Batch	Synthetic medium II with different salt concentrations of NaCl	N
98	Shake flask	Batch	Synthetic medium II with different salt concentrations of KCl	N
42	DASGIP	Batch	Additional components (betaine, Fe, P)	P
4.2 Fermentation conditions				
48	DASGIP	Batch	Different pH values: pH 6, 7.5, 8, 8.5 >> 7, 7.5, 8, 8.2 (changed)	P
51	Shake flask	Batch	Unsterile substrate without inoculum	P
63	Shake flask	Batch	Sterile and unsterile substrates with inoculum	P
4.3 Feeding strategies				
54	DASGIP	Fed-batch	4 time, 40 mL of feed shot (60 and 80%), 4 hours of interval	P
57	DASGIP	Fed-batch	4 times, 50 mL of feed shot (100%), 4 hours of interval	P
65	DASGIP	Fed-batch	4 times, 50 mL of feed shot (80%), 4 hours of interval	P
70	DASGIP	Fed-batch	5 times, 50 mL of feed shot (unsterile 80%)	P
94	DASGIP	Fed-batch	4 times, 50 mL of feed shot (unsterile 80%), 4 hours of interval	P

3.4 Analytical methods

3.4.1 Optical density

The optical density (OD) was measured at 600 nm by a DR 3900 Hach Lange photometer. Reverse osmosis water was used as blank. The valid measuring range was determined between 0.1 and 1 to ensure a linear dependence between density and measured signal. The samples were diluted with reverse osmosis water. All desugarized molasses samples were diluted by 1:50 to 1:200.

3.4.2 pH

The pH value of the samples taken from shaking flasks was measured by a pH meter (Mettler Toledo). If needed, the pH value was adjusted to around 7.

3.4.3 Fluorescence Microscope

To observe PHB under a fluorescence microscope, PHB was stained as described by Ostle *et al.* (1982). Nile blue A was used for staining, which was prepared in a concentration of 10 mg/mL by solving it at 70 °C for one hour, followed by filtrating with the filter paper. The sample was diluted to optical density (OD) of 0.5 and distributed on a microscope slide. After the suspension was dried, it was heat-fixated by a flame. The microscope slide was treated with Nile blue A at 55 °C for 10 min. During these 10 minutes, the slide was put in a petri dish to avoid the evaporation of the dye. Afterwards, the Nile blue A was rinsed with reverse osmosis water, followed by immersing of the slide in 8% acetic acid for 1 min. Once the microscope slide was dried, the stained cells were covered with a glass coverslip. Consequently, they were observed under a fluorescence microscope (Olympus Vanox AHBT3) with 60x magnification after having applied one drop of immersion oil.

3.4.4 Biomass (CDW)

5 mL of sample was put into a 10 mL preweighed pyrex glass tube and centrifuged at 2800 rcf for 15 minutes (Eppendorf, Centrifuge 5810). Afterwards, the supernatant was transferred into a falcon tube for desugarized sugar, nitrogen and phosphate analysis. The cell pellet was washed two times with reverse osmosis water (see Figure 4), then dried in the 105 °C drying oven for at least 48 h (Heraeus Instruments) until a constant weight was reached. The dried cell pellet was weighed with an analytical balance (MC1 Analytic AC 210S Sartorius).

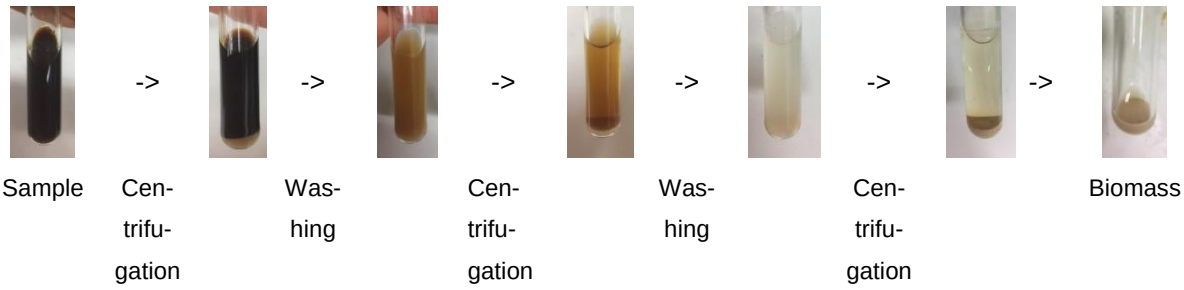


Figure 4: The steps to achieve biomass.

3.4.5 Polyhydroxyalkanoates (PHAs)

PHAs were determined by a modified method of Furrer *et al.* (2007). The dried cell pellet was dissolved in the solution A which included 2-ethyl-2hydroxybutyric acid as an internal standard, followed by adding the solution B after 1 hour. The tube was tightly sealed and

placed in a vortex mixer. Subsequently, it was placed in a water bath and heated at 80 °C for at least 16 hours. After cooling down to room temperature, 2 mL of ultrapure water was added and put in the vortex mixer. After phase separation the aqueous phase was removed with a Pasteur pipette. To remove the remaining water one spoonful of Na_2SO_4 and of Na_2CO_3 was added into the organic layer and mixture was vortexed. Lastly, the dehydrated organic layer was centrifuged at 2800 rcf for 15 minutes (Eppendorf, Centrifuge 5810). The supernatant was transferred into a vial and analyzed using gas chromatography with a flame ionization detector (Agilent Technologies 7890B). The gas chromatograph is equipped with an automatic injector (Agilent Technologies 7683B) and an Agilent 19091G-133:HP-35 column (30 m x 0.25 mm) with a thickness of 0.25 µm. The sample was injected in a split mode. The initial temperature of 50 °C, was raised with a rate of 15 °C min^{-1} to 150 °C, 10 °C min^{-1} from 151 to 200 °C, and 25 °C min^{-1} from 201 to 280 °C. Helium and nitrogen were used as a mobile phase and a makeup gas, respectively.

- Solution A: 10 mg/mL of 2-ethyl-2hydroxybutyric acid as an internal standard in Methylene chloride
- Solution B: 20/80 (v/v) mixture of HCl (37%) and iso-propanol

3.4.6 Sugars

For sugar analysis, the supernatant obtained after centrifugation was used. 33 µL sample was put into a 1.5 mL eppi tube and 67 µL invertase (10 mg/mL) (SIGMA, I9253) was added into it. After mixing it with a vortexer, the eppi tube was treated in the thermo block (Eppendorf thermomixer comfort) at 50 °C with 800 rpm for at least 30 min. 820 µL reverse osmosis water with a pH value of 4 was added. Afterwards, 40 µL of carrez reagent 1 and after mixing the samples and waiting for 5 min, 40 µL of carrez reagent 2 were added for protein precipitation. The samples were centrifuged at 12500 rpm (14324 g) for 20 min (BECKMAN, CS-15) to separate the precipitated proteins from the supernatant. Lastly, the sample was filtered by a 0.45 µm filter membrane and transferred into a vial. The desugarized sugar quantification was carried out through High Performance Liquid Chromatography (Agilent 1100 series) using an automatic injector (G1316A Hewlett Packard), a column (ICSep ICE-ION-300 Transgenomic) and a RI (Refractive Index)-detector. 0.01N sulfuric acid was used as a mobile phase at 0.375 mL min^{-1}.

- Carrez reagent 1: 5.325 g of $K_4[FE(CN)_6]\cdot 3H_2O$ in milli-Q water up to 50 mL
- Carrez reagent 2: 14.400 g of $ZnSO_4\cdot 7H_2O$ in in milli-Q water up to 50 mL

3.4.7 Total Kjeldahl Nitrogen (TKN) and Free Ammonia

To determine the total nitrogen, the Kjeldahl method, developed by Johan Kjeldahl, was used. The supernatant was weighed in a heat-resistant glass tube (Buechi) and one Kjeldahl tablet (1000 Kjeltabs CT) consisting of potassium sulfate, copper (II) sulfate and titanium dioxide was added to it as a catalyst. 20 mL of concentrated sulphuric acid were added and the glass tubes were placed in the digestion system (Büchi Digest Automat K-438). After the digestion process, the sample was cooled down to room temperature and analyzed with AutoKjeldahl unit (Büchi AutoKjeldahl Unit K-370). The amount of 0.05 N of hydrochloric acid consumed for back titration was used to calculate the amount of nitrogen in the sample.

- Digestion:
  $Sample + H_2SO_{4(l)} \rightarrow CO_{2(g)} + SO_{2(g)} + H_2O_{(g)} + (NH_4)_2SO_{4(aq)}$
- Distillation:
  $(NH_4)_2SO_{4(aq)} + 2NaOH_{(aq)} \rightarrow Na_2SO_{4(aq)} + 2H_2O_{(l)} + 2NH_{3(g)}$
  $NH_{3(g)} + B(OH)_{3(aq)} + H_2O \rightarrow NH_4^+{}_{(aq)} + B(OH)_4^-{}_{(aq)}$
- Back-Titration:
  $NH_4^+{}_{(aq)} + B(OH)_4^-{}_{(aq)} + HCl_{(aq)} \rightarrow NH_4Cl_{(aq)} + B(OH)_{3(aq)} + H_2O$

3.4.8 Phosphate

A premade phosphate kit (LCK 350 Phosphate – HACH) was used to determine total phosphorus present in the sample. The sample preparation was done according to Hach Lange LCK 350 phosphate test kit manual. The chemical reaction takes place as follows: phosphate ions in the sample react with molybdate and antimony ions in an acidic solution, resulting in the formation of an antimonyl phosphomolybdate complex. As the complex is reduced by ascorbic acid, a deeply blue-colored product (phosphomolybdenum blue) is formed, which can be determined by photometric measurement (DR 3900 HACH Lange photometer).

4 Results and Discussion

4.1 Media conditions

4.1.1 Effect of desugarized molasses on biomass and PHB production from different batches

Two different batches containing desugarized molasses were provided by the sugar manufacturing company. As the batches differ from each other in their composition, it was necessary to investigate the impact on the growth behavior. Those experiments were carried out in shake-flasks. *B. megaterium* showed similar growth behavior regardless of the desugarized molasses batch. The results are shown in Figure 5. As it can be seen a slightly higher biomass concentration of 11.8 g/L compared to 10.7 g/L was achieved with the batch 17. Interestingly a lower PHB formation was observed when using the batch 17 resulting in 6.2 g/L PHB compared to 7.1 g/L with the batch 16. The biomass and PHB concentrations were determined only once during the whole experiment, due to the low working volume in the shake flasks.

A detailed comparison can be seen in Table 13. The data indicates a higher yield of PHB per g sugar added when the batch 17 is used. The difference between the yields obtained with both batches is not remarkable. Considering the similar growth behavior of *B. megaterium*, it can be stated that the desugarized molasses from different batches has no significant impact on the biomass as well as PHB production. As only two batches were examined, however, further experiments with the desugarized molasses from other batches are recommended.

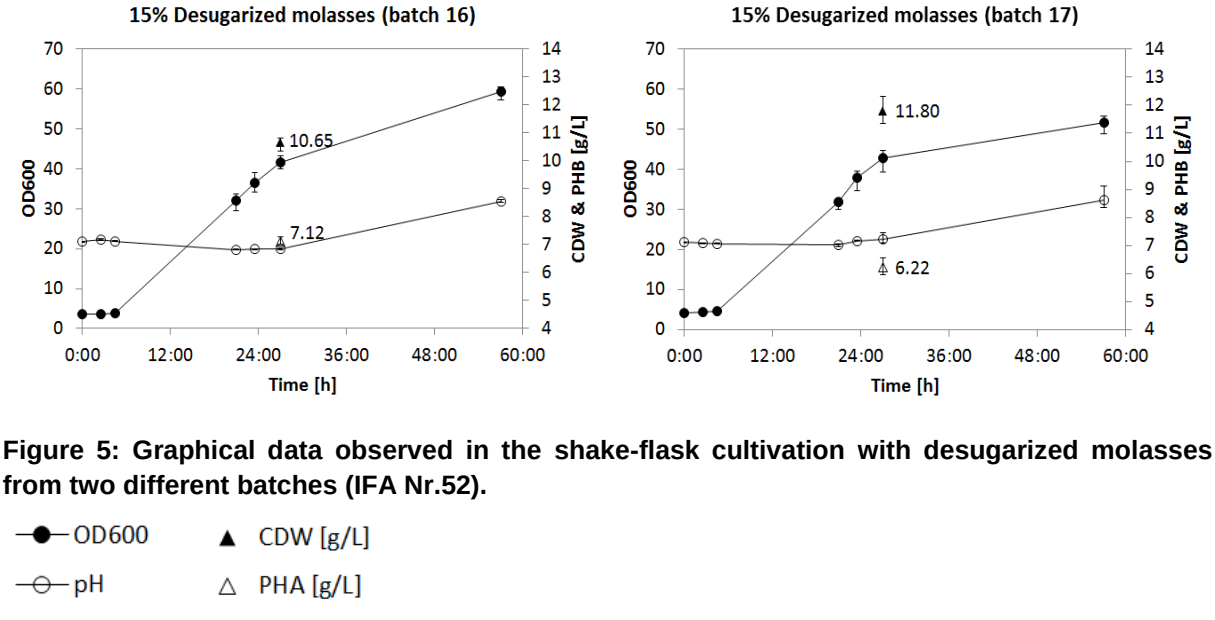


Figure 5: Graphical data observed in the shake-flask cultivation with desugarized molasses from two different batches (IFA Nr.52).

Table 13: Comparison between 15% desugarized molasses from two different batches (IFA Nr.52); Yields calculation is based on the sugar content in the desugarized molasses.

	15% DM from Batch (2016.05)	15% DM from Batch (2017.08)
PHB content [g PHB / g CDW]	0.67	0.53
Yield [g PHB / g sugar]	0.28	0.32

Subsequently to the low sugar concentration of batch 17 it was assumed that a higher desugarized molasses concentration can be used. In the next experiment various desugarized molasses concentrations from 17 to 40% were tested, in order to achieve maximum biomass and PHB formation. A summary of the results is shown in Figure 6. Generally, it can be said that if growth was observed, the pH became more acidic in the first few hours of the experiment and later it became alkaline (data shown in Annex Table A1). The pH change was caused by degradation of components present in the desugarized molasses. No growth was observed in desugarized molasses concentrations above 35%. With 30% desugarized molasses, the lag phase increased to 72 hours. In the concentrations between 15 to 25%, the changes in the optical density were faster and higher values were reached. Considering the maximum optical density as well as the time needed to reach it, 20% desugarized molasses was chosen for the further experiments.

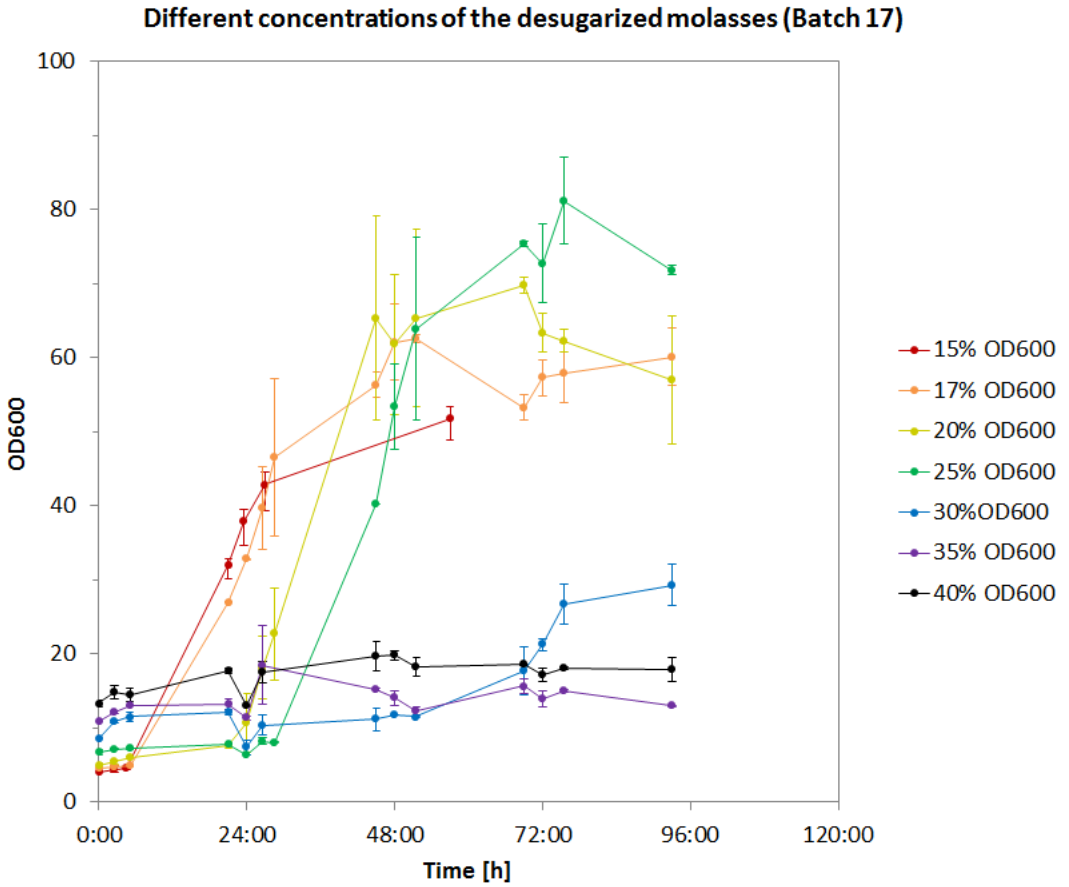


Figure 6: Graphical OD 600 data of different desugarized molasses concentrations (IFA Nr.47).

4.1.2 Essential and/or beneficial substances

4.1.2.1 Effect of different kinds of salt on biomass and PHB production

Research has shown that the use of molasses rather than synthetic medium led to higher concentrations of biomass and PHB (Shasaltaneh *et al.*, 2013; Gouda *et al.*, 2001; Purushothaman *et al.*, 2001). To find out which substances present in desugarized molasses are beneficial for biomass and PHB production, experiments with the synthetic media were performed. More specifically, possible enhancing components, namely different kinds of salt (potassium and sodium) and phosphorus, were investigated by adding them to media.

The two experiments were performed with the synthetic medium II, mentioned in chapter 3 (one with KCl and the other one with NaCl). It was observed that *B. megaterium* grew faster and better, when KCl was used for the medium instead of NaCl (see Figure 7). The comparison of the results between the media with KCl and NaCl, revealed a particularly significant difference in the media containing the lowest and the highest salt concentrations. Replacing NaCl with KCl resulted in a 1.5 times higher optical density at the lowest salt concentration (0.086M) and even more than double at the highest concentration (1.71M). Biomass and PHB formation were clearly higher in the media with KCl at all salt concentrations (see Table 14). The sugar was consumed in a similar manner regardless of the salt type, except in the salt concentrations of 1.28M and 1.71M.

Potassium (K) plays an important role as the major intracellular cation in bacteria. Moreover, potassium is a co-factor of some enzymes which are needed for certain chemical reactions such as the glycolysis pathway in bacteria (Gottschalk, 1986). In this regard, the presence of a high amount of potassium might increase the sugar consumption and might improve its efficiency; this might be the reason that *B. megaterium* accumulated more biomass as well as PHB.

Oren (1999) stated that most halophiles keep the osmotic balance either with the help of small organic molecules synthesized by cells or by maintaining a high salt concentration in the cytoplasm. The high salt (KCl) concentration of the media might have allowed for the high intracellular potassium concentration in *B. megaterium,* necessary for an osmotic equilibrium. This might be another reason that *B. megaterium* grew better in the media with the high potassium concentration than in the medium with the high sodium concentration.

B. megaterium was isolated from the salt lake Uyuni where more potassium was found compared to the other salinity environments such as seas or salt lakes (Grant, 2004; Javor, 1989; Perez-Fernandez *et al.,* 2016; Wielen *et al.,* 2005). The *B. megaterium* strain used in this study might have a preference for an environment with high concentration of potassium.

The increased amount of phosphate also contributed to enhancing the yield, when comparing the two-synthetic media 1 and 2 (see Table 14). Phosphorus (P) is one of the most important elements in the cells, as it is involved in the synthesis of nucleic acids, phospholipids and nucleotides such as ATP, GTP, NAD^+ and FAD (Gottschalk, 1986). Moreover, Ryu *et al.* (1997) reported that a sufficient phosphate supply in the growth phase can increase the cell growth and PHB production. Thus, it can be assumed that the increased amount of phosphate to 6.10 g/L can increase the cell growth for *B. megaterium*.

The presence of potassium (K) as well as the increased phosphorus (P) concentration contributed to a higher production of biomass and PHB. The results were still lower however, compared to the ones with desugarized molasses (see Figure 5 and Table 14). The reason for this is, that there are substances other than potassium and phosphorus, which have an influence on the cell formation; for example, organic nitrogen compounds, trace elements, vitamins or amino acids (Shasaltaneh *et al.*, 2013; Gouda *et al.*, 2001; Purushothaman *et al.*, 2001).

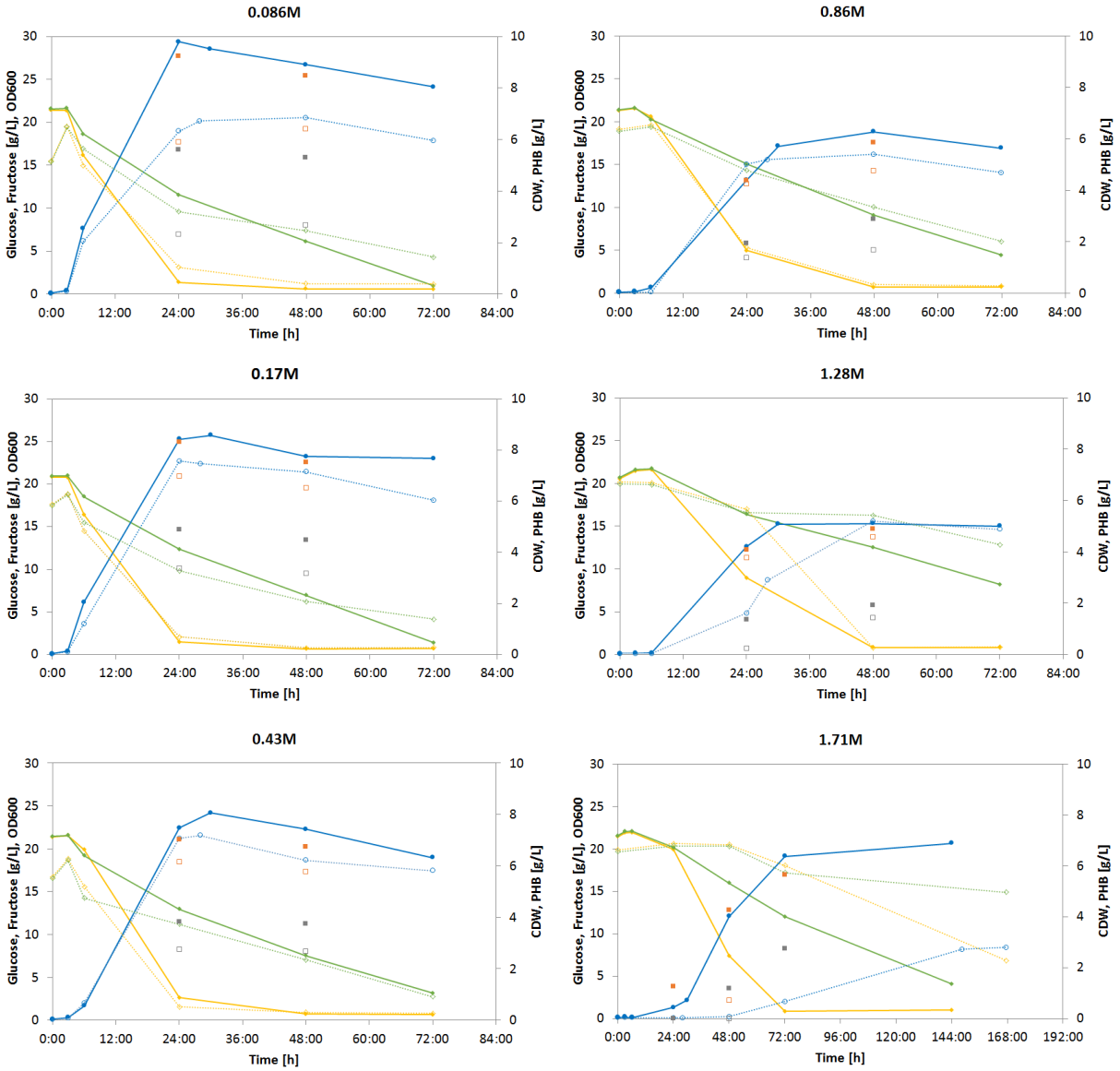


Figure 7: Graphical data obtained from synthetic media with KCl and NaCl (IFA Nr.91 and 98); The experiments were conducted in duplicate. The data presented are average values. The average and error range obtained from these two experiments can be found in the Annex Table A2.

Table 14: Comparison between synthetic media with KCl and NaCl; [1)] (I): contains 6.10 g/L phosphate (3.7 g/L KH_2PO_4, 7.5 g/L $K_2HPO_4 \cdot 3H_2O$, 0.6 g/L Na_2HPO_4); [2)] (II): contains 1.80 g/L phosphate (2 g/L KH_2PO_4, 0.6 Na_2HPO_4). The data shown was obtained from the experiments IFA Nr.98, 91 and 39.

	Synthetic media with KCl 0.17M	Synthetic media with NaCl 0.17M [1)]	Synthetic media with NaCl 0.17M [2)]
Duration [h]	48	48	40
CDW [g/L]	7.51	6.51	5.70
PHB [g/L]	4.48	3.16	2.06
PHB content [g PHB/g CDW]	0.60	0.49	0.36

4.1.2.2 Effect of an increased concentration of betaine, iron and phosphate

The formal experiments (IFA Nr.39 and 91) carried out with synthetic media revealed that more phosphate present in the media increased the biomass and PHB concentrations (Table 14). Thus, it was investigated in this study if additional phosphate also has an effect with desugarized molasses. Moreover, as the increased amount of iron (Fe^{2+}) to 0.025 g/L showed a better production of PHB in the previous study (data shown in Annex Table A3), the effect of iron addition was examined as well. The substrate collected in 2017 includes less betaine, since the sugar manufacturing company improved the extraction of betaine from molasses. For this reason, the impact of additional betaine on the growth behavior was investigated. To compare the impact of betaine, iron and phosphate on PHB production, 15% desugarized molasses without any additive was included in the experimental plan.

The results from the bioreactor cultivation are shown in Table 15. There was no significant difference between 15% desugarized molasses and 15% desugarized molasses with the addition of 4.3 g/L betaine and 0.025 g/L iron. An increased biomass formation and a faster sugar consumption was observed with the addition of phosphate, compared to the other three media (betaine, iron, without any additives). After 48 hours all the sugar was consumed in all four experiments. Interestingly *B. megaterium* is first consuming glucose, then lactic acid and at last fructose (data shown in Annex Figure A1).

Although more biomass was produced in a shorter time, *B. megaterium* accumulated less PHB in the presence of a higher phosphate concentration. This was to expect as phosphate limiting conditions were not or only at the end of the fermentation achieved. If there had been enough carbon sources, more biomass as well as PHB concentrations would probably have been obtained. To verify this assumption, fed-batch cultivation was carried out in the medium including the additional phosphate. The relevant data of these experiments can be found in chapter 4.1.3.2 Phosphorus.

The other three media conditions, namely 15% desugarized molasses, 15% desugarized molasses with the addition of betaine and iron, showed a similar growth behavior and PHB concentration. Kotzamanidis *et al.* (2002) reported that the abundant presence of metals (e.g. iron, zinc, magnesium and calcium etc.) in the medium inhibits the growth of microorganisms, as they inactivate the enzymes. In contrast, trace elements including the above mentioned metals as well as chloride and sulfur etc. are essential for bacterial growth (Kanjanachumpol *et al.,* 2013). If too many of these trace elements are present, the counter effect might occur though. The amount of added iron or betaine might not have been enough in this study to cause any changes. To find out the effect of these components, further experiments with different concentrations are recommended.

It has to be mentioned that the above experiment was carried out without duplication, hence the data is not fully reliable. It serves as a rough estimate to determine whether there is a change in the growth behavior and PHB production, depending on the different substrate conditions.

Table 15: Comparison between 15% desugarized molasses and the additional substances (IFA Nr.42).

	15% DM	**15% DM + 4.3 g/L Betaine**	**15% DM + 0.009 g/L $FeSO_4$**	**15% DM + 0.133 g/L PO_4^{3-}**
Duration [h]	24	24	24	24
CDW [g/L]	17.18	15.88	14.52	19.46
PHB [g/L]	10.22	9.80	8.81	8.19
PHB content [g PHB/g CDW]	0.59	0.62	0.61	0.42
Yield [g CDW/g DM added]	0.14	0.13	0.12	0.16
Yield [g PHB/g DM added]	0.085	0.082	0.073	0.068

4.1.3 Nutrients consumption

PHB production by microorganisms occurs under stress conditions such as nitrogen, phosphorus or oxygen limitation (Marina *et al.,* 2017; Reddy *et al*., 2003; Salgaonkar *et al.*, 2013; Wang *et al*., 2014). Thus, total nitrogen and phosphorus concentrations were analyzed to see if their concentrations have an impact on the PHB production.

4.1.3.1 Nitrogen

The nitrogen concentrations were determined during the fermentation processes, as described in chapter 3.4.7 Total Kjeldahl Nitrogen (TKN) and Free Ammonia.
The concentrations showed a steady decrease over time. When feed was added to the medium however, a sudden increase could be observed due to the nitrogen present in the feed.
With an initial sugar concentration of 30 g/L and initial TKN (Total Kjeldahl Nitrogen) concentration of 3.74 g/L (batch) and 3.52 g/L (fed-batch), the C/N ratios of the batch and the fed-batch cultivations are 8 and 8.5, respectively. According to Kulpreecha *et al.* (2009) enhanced cell growth and PHB production were observed when the C/N ratio was 25. Kanjanachumpol *et al.* (2013) reported similarly that more PHB production was achieved with a 12.5 C/N ratio compared with a 10 C/N ratio. Compared to the figures cited above, the C/N ratios in the experiments carried out in this study were comparatively low. The low C/N ratio results in nitrogen limitation to be unfeasible when desugarized molasses is used.

4.1.3.2 Phosphorus

Table 16 shows the comparison between the desugarized molasses with and without the addition of phosphate. When phosphate was added, a higher biomass concentration with a lower PHB content was achieved in both batch and fed-batch fermentations. The reason for this might be that the synthesis of PHB is favored by phosphorus limitation with the desugarized molasses, since there is a sufficient amount of nitrogen present in the desugarized molasses. The additional phosphate improves only the biomass production when desugarized molasses is applied as a medium. It can be stated that adding phosphate to the desugarized molasses medium is suitable for enhancing the growth of *B. megaterium* but not helpful for the PHB production.

The data below (Table 16) is based on the amount of phosphate added to the media. To determine the phosphate, the phosphate kit (LCK 350 Phosphate – Hach-Lange) was used. The data-, obtained from this kit-, reflected the increase and decrease of the phosphate content. The reason for this was that the feed containing phosphate was added to the media and that the phosphate was consumed by *B. megaterium*. However, the exact amount of

phosphate present in the media could not be determined. The chemical reaction might either have been inhibited by unknown components in the desugarized molasses or the brown color of the desugarized molasses might have disturbed the measurement of the photometer. Further studies are recommended to develop a better determination method of phosphate in order to find out the correlation between the phosphate concentration in the desugarized molasses and the PHB accumulation.

Table 16: Comparison between the desugarized molasses with and without the additional phosphate (IFA Nr.42, 57 and 65). DM stands for desugarized molasses. Each experiment was performed in duplicate. This table shows only the average value. The single data is shown in Table 20.

Substrate Batch	Feed	Phosphate addition	Total phosphate [g/L]	CDW [g/L]	PHB [g/L]	PHB content [g PHB /g CDW]
15% DM	-	No	0.13	17.18	10.22	0.59
15% DM	-	Yes	0.15	19.54	8.60	0.44
20% DM	80% DM	No	0.30	50.47	44.41	0.88
20% DM	80% DM	Yes	0.37	56.88	47.45	0.83
20% DM	100% DM	No	0.34	27.80	15.49	0.56
20% DM	100% DM	Yes	0.48	31.12	14.79	0.48

4.2 Fermentation conditions

4.2.1 Effect of various pH values on biomass and PHB production

In order to determine the optimum pH value for growth and PHB formation with *B. megaterium*, experiments with various pH values, ranging from 6 to 8.5.

As seen in Figure 8, there was no growth below pH 6 and above pH 8.5. Once the pH values were changed to 7 and 8.2 respectively, *B. megaterium* started sugar consumption immediately. With pH 7 the maximum biomass (27.1 g/L) as well as PHB (13.6 g/L) concentration was achieved. Experiments with pH 7.5 and 8 showed similar results. When the pH was regulated to 8.2, the biomass and PHB production was not as high as in the other conditions.

B. megaterium could survive below pH 6 and above pH 8.5, but no growth was observed under these conditions. According to these results the optimum pH value is between 7 and 8.2 for PHB production by *B. megaterium*. A possible reason might be that 7.0 is the optimal pH value of the enzymes involved in the PHB biosynthesis pathway (Kulpreecha *et al.,* 2009). In this study, the highest PHB content, yield (g PHB/g sugar) and productivity were achieved when the pH of the media was controlled at 7.0.

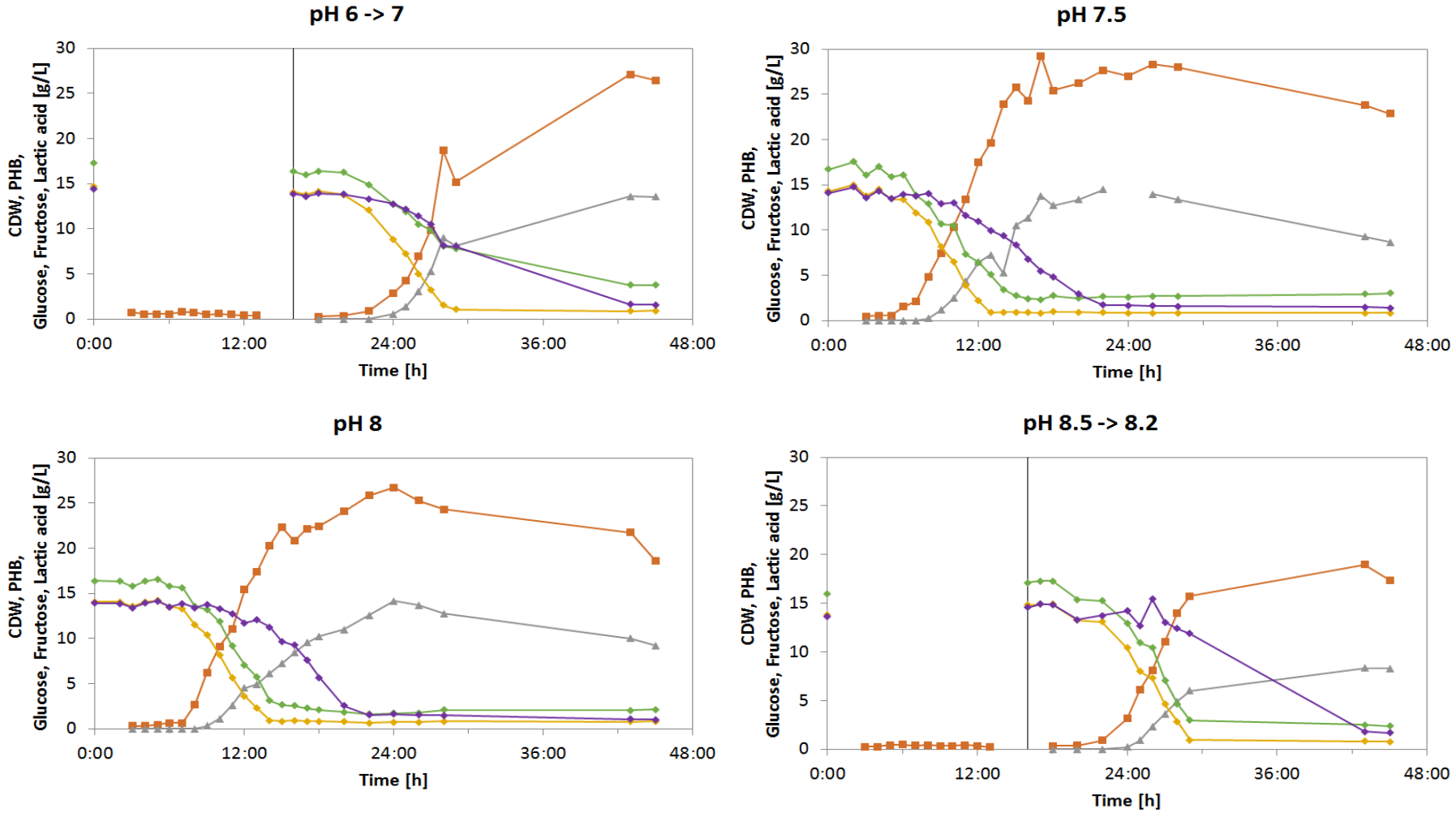


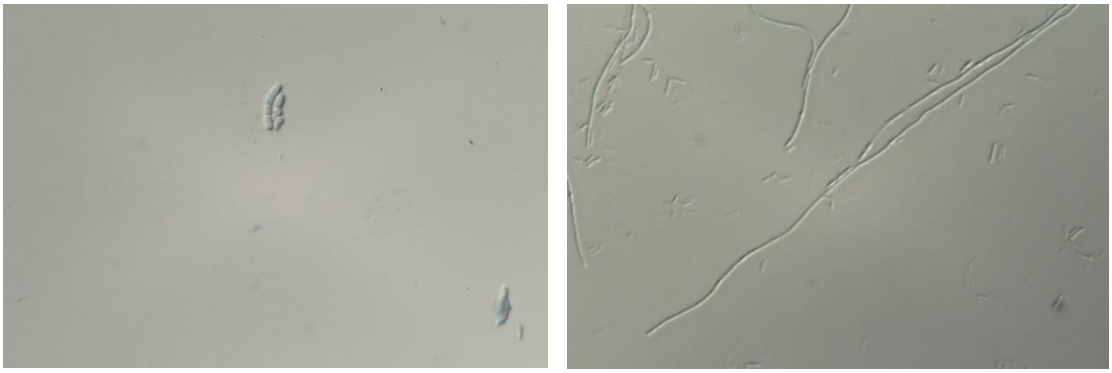
Figure 8: Graphical data of the experiment with the different pH values (IFA Nr.48).

4.2.2 Effect of fermentation on biomass and PHB production under unsterile conditions

Concentrated desugarized molasses has a relatively low water activity (a_w), due to its high dry mass of 72%, making it stable for long time storage. Due to the lack of active organism under these conditions it was assumed that sterilization might not be necessary, offering a possibility to reduce production costs. In order to examine this assumption pasteurized and unsterile desugarized molasses was used.

First, the desugarized molasses treated with different pasteurization methods was fermented in the shaking flasks. In order to reveal possible microorganisms that grow in the desugarized molasses (when diluted), the experiments were not inoculated with *B. megaterium*.

Based on the observation under microscope, the morphology of the micoorganisms observed, was different in comparison to *B. megaterium* (Picture 4). The main changes in the optical density and pH values were noted during the first 96 hours in all the flasks (see Figure 9). This is a considerably longer time compared to *B. megaterium* which reaches its maximum optical density after 48 hours.



Picture 4: *Bacillus megaterium* (left), other microorganisms grown in the unsterile molasses (right).

Considering the different shapes of microorganisms detected on the microscopic slide, it was proven that other microorganisms were growing in the unsterile desugarized molasses. They could not be eliminated by any heat treatments, either heating up to 60 °C for 30 min or to 95 °C for 2 min. It is fair to assume that thermophilic microorganisms survive the sugar production process and are inactive in the concentrated desugarized molasses. The other microorganisms were growing quite slowly, compared to the 2 to 5 hours of lag phase of *B. megaterium*. Thus, there is reason to believe that other microorganisms might be outgrown by *B. megaterium* under unsterile conditions.

To prove the assumption mentioned above, unsterile desugarized molasses was inoculated with the pre-culture of *B. megaterium*, growing on BX medium (nutrient rich medium) and sterile desugarized molasses was prepared as a positive control.
In the flasks containing the sterile desugarized molasses, the optical density was more than doubled, indicating more biomass production compared to the unsterile substrate (data shown in Annex Figure A2). In the unsterile desugarized molasses no PHB was produced, whereas *B. megaterium* accumulated 4.41 g/L of PHB with 0.29 g PHB per g CDW in the sterile desugarized molasses (see Table 17).

In unsterile conditions, *B. megaterium* could not grow, therefore no PHB production was observed. When the desugarized molasses was diluted with reverse osmosis water, the water activity increased and microorganisms present in the desugarized molasses could start growing. However, considering the lower optical density, they could not grow well. The results match with the observation of the results above. These thermophilic organisms might need a higher temperature for optimal growth. Since there was no PHB production observed in the unsterile condition, it is assumed that the growth of *B. megaterium* was inhibited by the presence of other microorganisms. Thus, it is not desirable to use unsterile desugarized molasses to produce PHB.

The unsterile fermentation process can contribute not only to save sterilization energy, but also reduce fermentation complexity, thereby improving process effectiveness (Wang *et al.*, 2014). Some studies have investigated PHB production with extremophile microorganisms under unsterile conditions. These harsh environments can prevent the unwanted microbial growth of other microorganisms than extremophiles. *Haloferax mediterranei* DSM1411, which grows in a salt concentration from 1.5M to 5M NaCl, can produce PHB (Lillo and Rodriguez-Valera, 1990). Moreover, Tan *et al.* (2011) obtained 40 g/L of CDW with 60% of PHB content of CDW by halophile, *Halomonas* TD01 in an unsterile process.
In former experiments, *B. megaterium* produced biomass as well as PHB at 1.71M of KCl. Thus, the combination of unsterile desugarized molasses with the higher salt (KCl) concentration might enhance the PHB production, as the high salt concentration inhibits the growth of other microorganisms.

It was assumed that once *B. megaterium* has a strong growth in the medium, it might continue growing even though unsterile substrate is applied. For this purpose, fed-batch cultivation with the sterile batch and unsterile feed was carried out. The data of this fed-batch cultivation can be found in chapter 4.3 Feeding strategies.

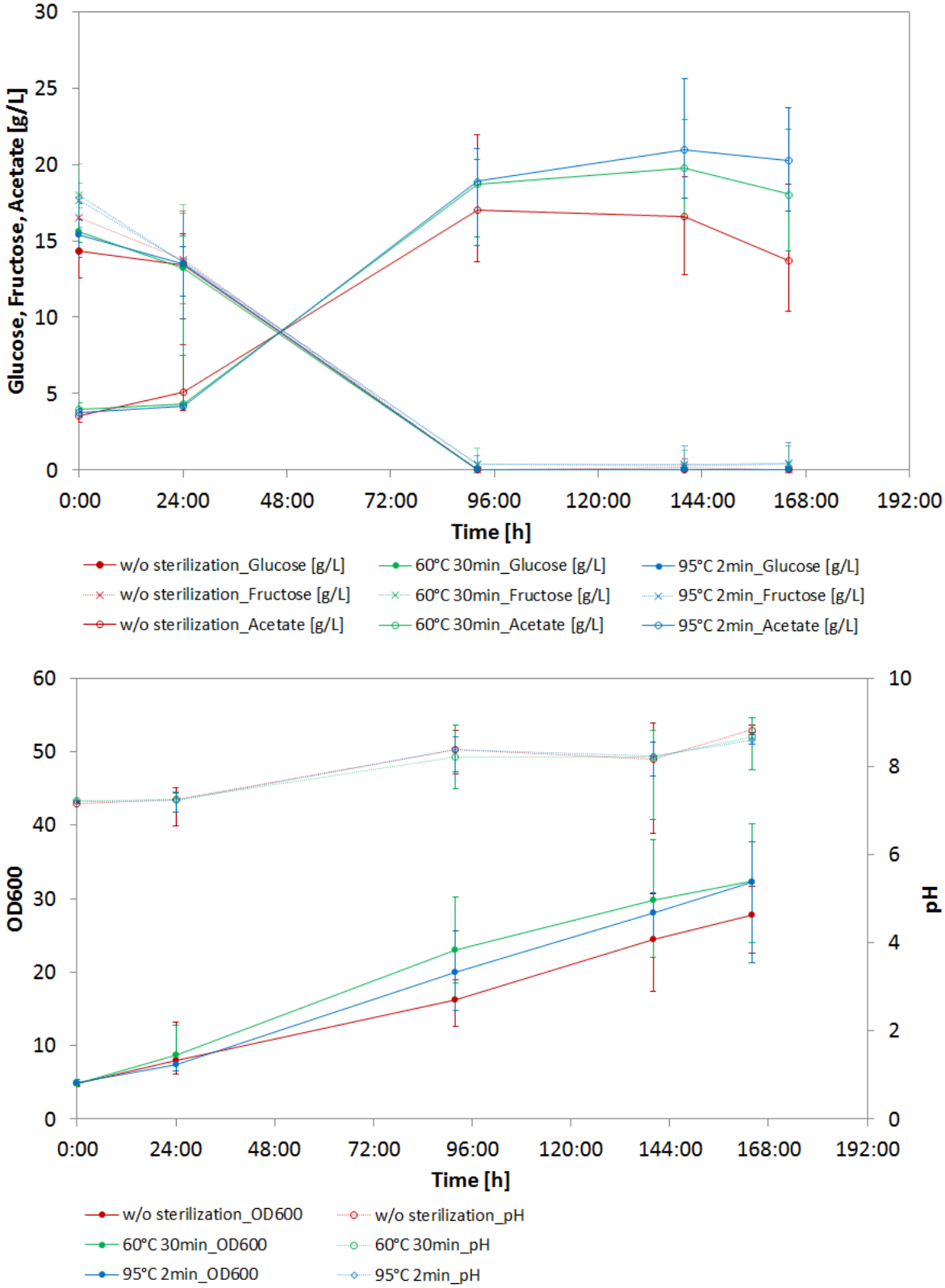


Figure 9: Graphical data of the experiment with the different heat treatments (IFA Nr.51).

Table 17: Comparison between unsterile and sterile desugarized molasses (IFA Nr.63).

	Unsterile desugarized molasses	Sterile desugarized molasses
Duration [h]	47.5	47.5
CDW [g/L]	2.44	15.32
PHB [g/L]	0.00	4.41
PHB content [g PHB / g CDW]	0.00	0.29

4.3 Feeding strategies

In order to achieve maximum PHB concentration as well as to enhance the yield of PHB, the fed-batch cultivation was performed with the so-called “feed shot” method (Purushothaman *et al.*, 2001; Sun *et al.*, 2007).

4.3.1 Sterile feed shot

Three fed-batch fermentation experiments (IFA Nr.54, 57 and 65) with 60, 80 and 100% (w/w) of sterile desugarized molasses were carried out according to the method shown in Table 12.

As shown in Figure 10, *B. megaterium* first used glucose and lactic acid, then fructose. Fructose was not consumed as fast as other sugars. In addition, the concentration of fructose was slightly higher than that of glucose at the beginning of the fermentation.

The interesting observation in these experiments is that after 10 hours of fermentation, *B. megaterium* already started accumulating PHB and its amount steadily increased. Furthermore, once the maximum PHB concentration reached the highest point, the PHB concentration remained rather constant for hours. Subsequently, it started declining however. The experiment with the 60% feed shot lasted only 37 hours, since the dissolved oxygen values were relatively stable indicating no significant growth of *B. megaterium*. On the other hand, when the 80 and 100% feed shots were applied, the fermentations were carried out for a longer period, in order to examine any further change in the biomass and PHB concentrations. It should be mentioned that the dissolved oxygen values could not be monitored for experiment IFA Nr.57 due to a calibration error. The agitation for this experiment was consequently regulated at 1100 rpm after 10 hours of fermentation.

Among three different concentrations of feed shot experiments the highest biomass production (50.47 g/L) was achieved with the 80% feed shot. The same was true for the PHB concentration (22.21 g/L). In comparison, the 60% feed shot and the concentrated feed shot led to lower results. Regarding the concentrated feed shot, it was noted that the biomass and PHB concentration slightly decreased after the fourth feeding

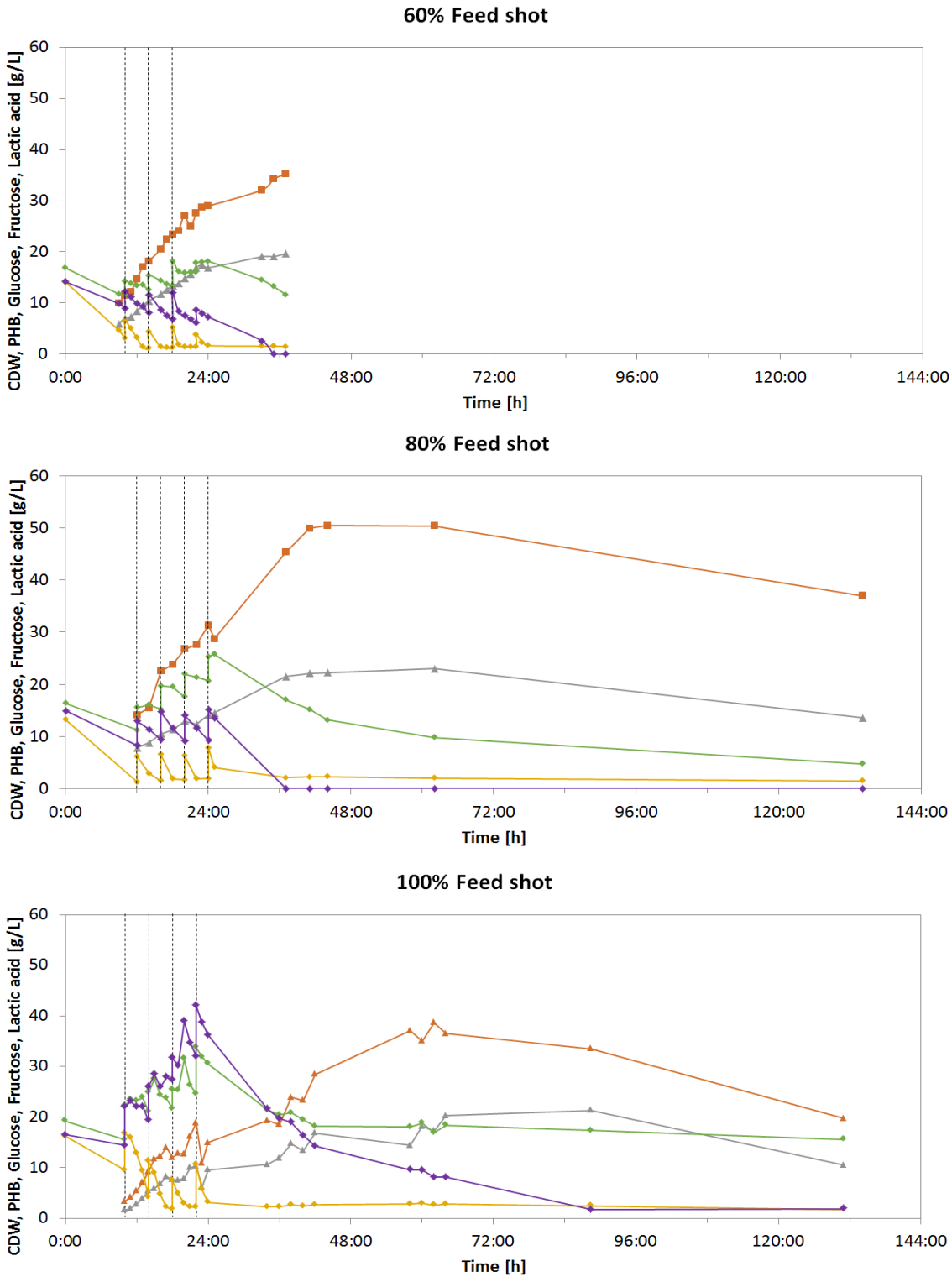


Figure 10: Graphical data of the experiments with the different feed concentrations (IFA Nr.54, 57 and 65). The experiments were performed in duplicate. This graph shows only the average value except the experiment with the undiluted feed shot.

Since sucrose consists of one molecule of glucose and fructose, the glucose and fructose concentrations should be the same at the beginning of the fermentation. However, it was observed that the higher the feed concentration was, the more desugarized fructose was detected. It can be assumed that there was a yet unknown component, with the same retention time at the HPLC as fructose, which was not consumable by *B. megaterium*. It was accumulated by adding more feed.

The PHB production started after 10–12 hours of fermentation. This behavior was observed in other studies working with *Bacillus megaterium* strains (Chaijamrus and Udpuay, 2008; Kanjanachumpol *et al.*, 2013; Kulpreecha *et al.*, 2009; Omar *et al.*, 2001; Rodríguez-Contreras *et al.*, 2013; Rodríguez-Contreras *et al.*, 2016; Salgaonkar *et al.*, 2013). *B. megaterium* might belong to the group of bacteria, which accumulate PHB without any nutrient limitation (Muhammadi *et al.*, 2015). As *B. megaterium* grows rapidly, phosphate gets quickly consumed and thus is already limited 10 hours after inoculation; which allows the *B. megaterium* to produce PHB at that point.

As the PHB concentration decreased after 72 hours (with the 80% feed shot) and after 96 hours (with the undiluted feed shot) of the fermentation, it can be said that the fermentation has to be stopped before the decrease of the PHB concentration (Valappil *et al.*, 2007b).

When the undiluted desugarized molasses was used as a feed, a decrease of the biomass and PHB concentrations after the fourth feeding was observed. This might be caused by the high sugar concentration inhibiting the growth of *B. megaterium.* It started growing again, once the sugar concentration reached an acceptable level. The inhibition of the growth explains that the highest biomass from the undiluted feed shot is lower than that of the fed-batch fermentation with the 80% feed shot, even though more carbon sources were added. The total sugar concentration was around 50 g/L after adding the last feeding. If the lactic acid concentration is also considered, more than 70 g/L of carbon sources were in the media. The study demonstrated by Kanjanachumpol *et al.* 2013 showed that the sugar concentration remained rather constant around 40 g/L in the fed-batch fermentation. In another study, the feed was applied to maintain the sugar concentration of 10 g/L (Valappil *et al.*, 2007b). Sufficient carbon sources can enhance PHB production. More than 40 g/L of sugar concentration, however, might inhibit *B. megaterium*.

After the experiments with the different concentrations of feed shot, it can be said that the concentrated feed shot might inhibit the growth, thus discouraging the accumulation of PHB. Therefore, the 60 or 80% feed shot is proposed for the fed-batch cultivation. On the other hand, while the undiluted feed shot was applied, the more sugar was clearly added to the

batch. Thus, it would be interesting to compare PHB formation between the different concentrations of feed shot, adding the same amount of sugar.

Comparing batch and fed-batch results gives a better understanding of the effectiveness of the PHB production (Table 18). The biomass and PHB productions were doubled in the fed-batch cultivation, compared to the batch fermentation. However, it took twice the time to reach these concentrations. Considering the cultivation time, higher PHB productivity was observed in the batch fermentation. This was shown in other studies as well. For example, the *Bacillus megaterium* BA-019 could produce more than double the amount of biomass and PHB but required longer fermentation time, when the fed-batch fermentation was applied (Kanjanachumpol *et al.*, 2013; Kulpreecha *et al.*, 2009).

Another advantage of batch cultivation is that a higher PHB content of CDW can be achieved. This is preferable as it leads to less biomass waste after the extraction (da Silva et al., 2018). Moreover, the higher PHB content of CDW also results in higher yields.

Overall, the batch cultivation is recommended to produce PHB from desugarized molasses by *Bacillus megaterium* CECT 7922, taking into consideration the higher productivity, PHB storage rate in the cells and the yield.

The yields shown in Table 18 have been calculated in three different ways (g sugar used, g sugar added as well as g desugarized molasses added). The following example shows how these values have been calculated in detail:
For IFA Nr.65 experiment, 600 mL of 20% desugarized molasses as an initial substrate and 200 mL of 80% desugarized molasses as a feed were applied. The density of water and of the desugarized molasses was assumed as 1 g/L. Only glucose and fructose were considered as sugar.

- g desugarized molasses added = (600 mL * 0.2) + (200 mL * 0.8) = 280 mL = 280 g
- g sugar used = g sugar added – [(g glucose + g fructose) in desugarized molasses at the end of the fermentation] = 66.6 g – 15.7 g = 50.9 g
- g sugar added = [(g glucose + g fructose) in initial desugarized molasses] + [(g glucose + g fructose) in feed desugarized molasses] = 27.2 g + 39.4 g = 66.6 g

In batch and fed-batch cultivations much higher CDW and PHB yields based on sugar (both used and added) were observed than with desugarized molasses. This can be explained by the fact that desugarized molasses, besides glucose and fructose, contains other carbon sources such as lactic acid and acetic acid. These other sources can also be used by the microorganism for biomass and PHB production.

Table 18: Comparison between the batch and the fed-batch cultivations; DM stands for desugarized molasses.

	Batch	Fed-batch
Duration [h]	24.75	48.24
CDW [g/L]	20.995	38.816
PHB [g/L]	11.028	19.260
[g PHB / g CDW]	0.525	0.496
PHB Productivity [g/L h]	0.446	0.399
Yield [g CDW / g sugar used]	0.923	0.580
Yield [g CDW / g sugar added]	0.708	0.447
Yield [g CDW / g DM added]	0.149	0.145
Yield [g PHB / g sugar used]	0.489	0.290
Yield [g PHB / g sugar added]	0.376	0.223
Yield [g PHB / g DM added]	0.079	0.072

4.3.2 Unsterile feed shot

In the partially unsterile condition, the fed-batch cultivation was executed. More specifically, only unsterile desugarized molasses was fed into the sterile substrate in the sterile bioreactor. There was a significant difference between the two parallel bioreactors. The concentrations of the biomass and the PHB were higher in one bioreactor with 36.20 g/L and 21.65 g/L, respectively. The maximum biomass and PHB concentrations achieved in the other bioreactor were 16.16 g/L and 8.4 g/L, respectively (see Figure 11).

As shown in Figure 12, lactic acid and acetic acid were produced in the second bioreactor. Thus, a higher base consumption was needed to maintain the pH 7.

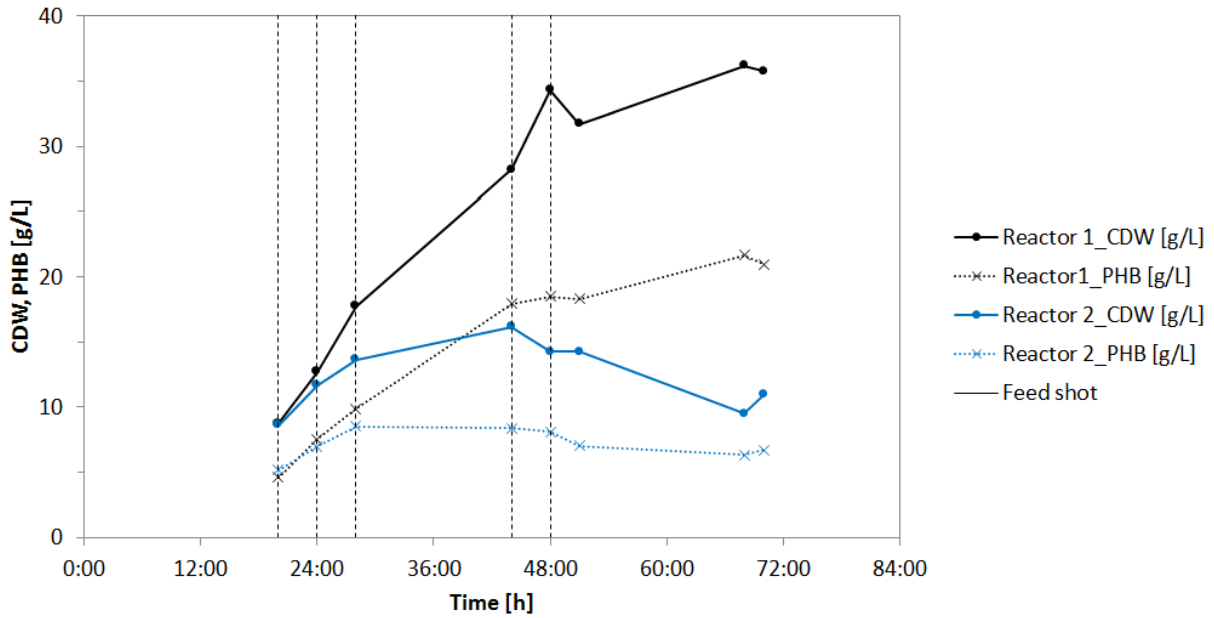


Figure 11: CDW and PHB concentrations under the partially unsterile condition (IFA Nr.70).

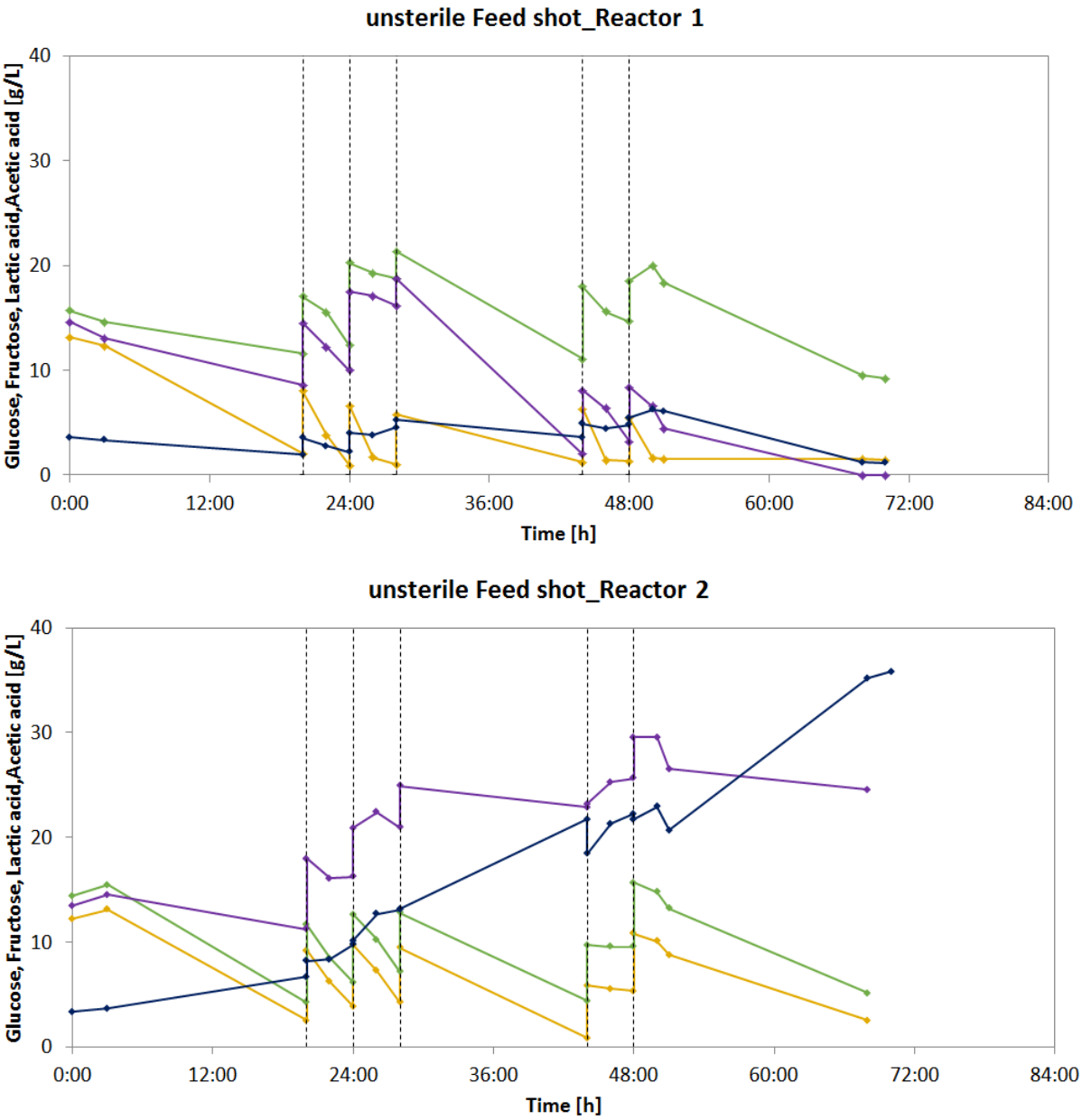


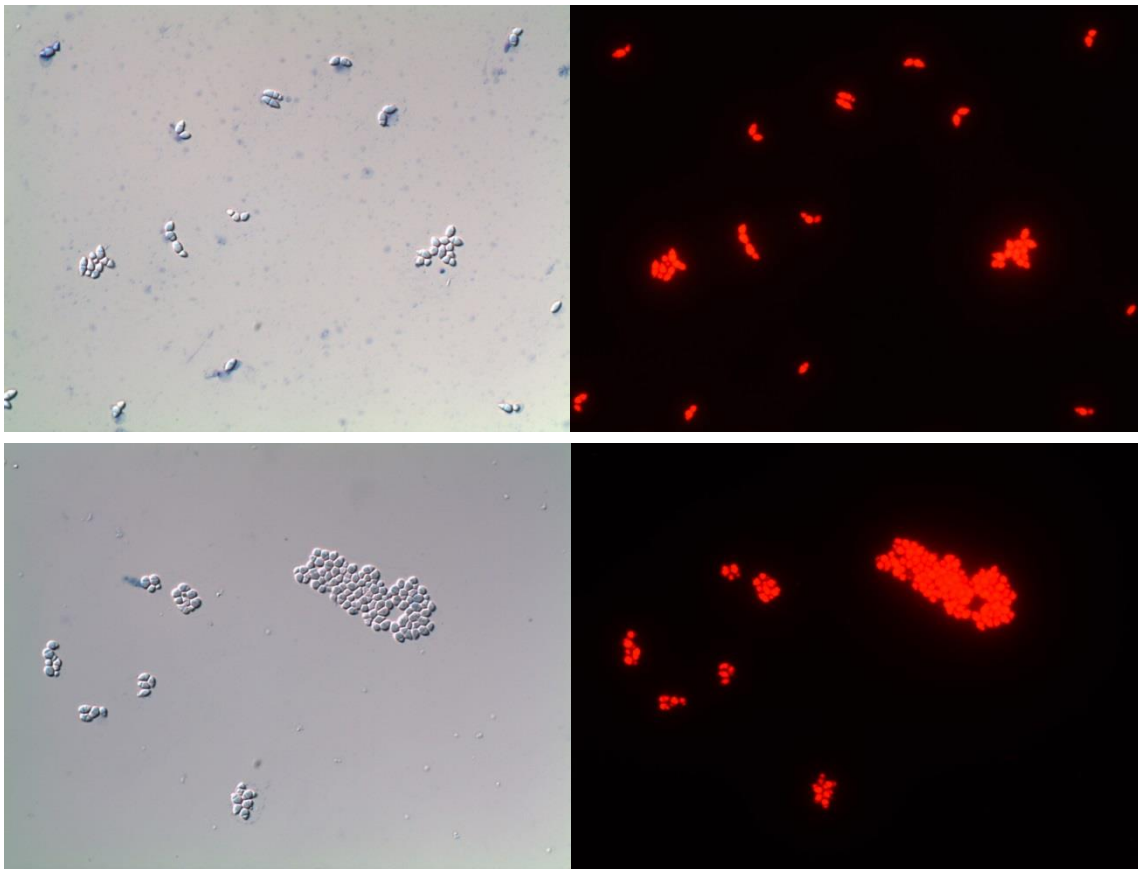
Figure 12: Sugar consumption during the fermentation under the partially unsterile condition (IFA Nr.70).

It is assumed that a significant difference in the results between two reactors is caused by the contamination. Other microorganisms, competing against *B. megaterium* for more carbon source, might have been more present in the feed shot prepared for the second bioreactor. Another possibility could be that the growth of *B. megaterium* in the second bioreactor was not as strong as the first one, when the feed shot was applied.

The production of lactic acid and acetic acid might be explained by an induced anaerobic condition, as other aerobic microorganisms used all the oxygen provided.

This experiment opened the possibility of PHB production despite contamination of the medium. To verify if the fed-batch cultivation with the unsterile feed is reproducible, the fed-batch fermentation under a partially unsterile condition was repeated.

Picture 5 shows the microscopic observation with the sample taken from experiment IFA Nr.94. It proves that *B. megaterium* was growing in the media. With fluorescence, the PHB was observed as it released a strong orange-red fluorescence.



Picture 5: Fluorescence microscopic observation: *Bacillus megaterium* taken from the experiment with the unsterile feed (IFA Nr.94) (left: *Bacillus megaterium* without fluorescence; right: with fluorescence).

The maximum biomass and PHB concentrations reached 41.02 g/L and 16.89 g/L, respectively with the 60% of the desugarized molasses feed, compared to 38.38 g/L and 19.05 g/L, respectively with the 80% of the desugarized molasses feed. Moreover, after 39 hours of fermentation, the PHB concentration remained steady in both conditions. The undefined substance was also detected at the end of the fermentation in this experiment (data shown in Annex Figure A3). The biomass and PHB produced with the 80% desugarized molasses feed shot were almost the same as that of the bioreactor with the 60% feed shot, even though more carbon sources, namely glucose, fructose and lactic acid, were added by the 80% feed shot.

Table 19 presents the comparison between the unsterile and the sterile feed shot. The fermentations with the unsterile feed shot proved that once *B. megaterium* shows strong growth, it continues growing and accumulates PHB despite the existence of the other microorganisms. However, the productivity and the yield were lower, compared to the fed-batch fermentation with the sterile feed. More research has to be carried out to produce PHB from desugarized molasses with *Bacillus megaterium* CECT 7922 in unsterile fermentation processes. It is for example recommended to repeat the experiment with unsterile feed to increase the reproducibility of the PHB production.

Table 19: Comparison between the unsterile and the sterile feed shot experiments.

	Fed-batch with sterile feed	**Fed-batch with unsterile feed**
Duration [h]	44	58.67
CDW [g/L]	50.470	36.013
PHB [g/L]	22.205	19.920
[g PHB / g CDW]	0.440	0.553
PHB Productivity [g/L h]	0.505	0.340
Yield [g CDW / g sugar used]	0.772	0.450
Yield [g CDW / g sugar added]	0.596	0.379
Yield [g CDW / g DM added]	0.180	0.133
Yield [g PHB / g sugar used]	0.340	0.252
Yield [g PHB / g sugar added]	0.262	0.212
Yield [g PHB / g DM added]	0.079	0.074

Table 20 presents the data of the CDW and PHB concentrations, the PHB productivity, the yields of CDW and PHB based on the desugarized molasses added (raw material) as well as the PHB content (of the CDW) along with the substrate used, namely the batch and feed, and the IFA experiment number.

Table 20: Data obtained from the experiments performed in this study; DM stands for desugarized molasses. The total desugarized molasses added was calculated on the assumption that the density of water and of the desugarized molasses is 1 g/L.

IFA Nr.	Culti-vation mode	Substrate		Cul-tiva-tion time	CDW	PHB	PHB Produc-tivity	CDW Yield	PHB Yield	PHB content
		Batch	Feed	[h]	[g/L]	[g/L]	[g/L h]	[g/g DM]	[g/g DM]	[gPHB /gCDW]
94	fed-batch	sterile 20% DM	unsterile 60% DM	45	36.25	18.89	0.42	0.16	0.08	0.52
94	fed-batch	sterile 20% DM	unsterile 60% DM	45	33.42	14.09	0.31	0.15	0.06	0.42
94	fed-batch	sterile 20% DM	unsterile 80% DM	63	36.43	19.56	0.31	0.13	0.07	0.54
94	fed-batch	sterile 20% DM	unsterile 80% DM	45	35.41	18.55	0.41	0.13	0.07	0.52
70	fed-batch	sterile 20% DM	unsterile 80% DM	68	36.20	21.65	0.32	0.13	0.08	0.60
65	fed-batch	sterile 20% DM	sterile 80% DM	44	49.08	21.16	0.48	0.18	0.08	0.43
65	fed-batch	sterile 20% DM	sterile 80% DM	44	51.86	23.25	0.53	0.19	0.08	0.45
65	fed-batch	sterile 20% DM+P	sterile 80% DM	44	54.32	23.17	0.53	0.19	0.08	0.43
65	fed-batch	sterile 20% DM+P	sterile 80% DM	44	59.44	24.28	0.55	0.21	0.09	0.41
57	fed-batch	sterile 20% DM	sterile 100% DM	60	35.04	18.17	0.30	0.11	0.06	0.52
57	fed-batch	sterile 20% DM	sterile 100% DM	60	20.56	12.80	0.21	0.06	0.04	0.62
57	fed-batch	sterile 20% DM+P	sterile 100% DM	60	31.56	15.72	0.26	0.10	0.05	0.50
57	fed-batch	sterile 20% DM+P	sterile 100% DM	60	30.68	13.86	0.23	0.10	0.04	0.45
54	fed-batch	sterile 20% DM	sterile 60% DM	35	34.44	18.84	0.54	0.15	0.08	0.55
54	fed-batch	sterile 20% DM	sterile 60% DM	33	31.44	19.34	0.59	0.14	0.09	0.62
54	fed-batch	sterile 20% DM	sterile 80% DM	35	40.68	21.25	0.61	0.16	0.08	0.52
54	fed-batch	sterile 20% DM	sterile 80% DM	35	43.06	22.84	0.65	0.17	0.09	0.53
48	batch	sterile 20% DM (pH7)	-	27	27.10	13.62	0.50	0.17	0.09	0.50
48	batch	sterile 20% DM (pH7.5)	-	22	27.68	14.48	0.66	0.17	0.09	0.52
48	batch	sterile 20% DM (pH8)	-	24	26.74	14.14	0.59	0.17	0.09	0.53
48	batch	sterile 20% DM (pH8.2)	-	27	18.96	8.34	0.31	0.12	0.05	0.44
42	batch	sterile 20% DM	-	24	17.18	10.22	0.43	0.14	0.09	0.59
42	batch	sterile 20% DM+Betaine	-	26	15.74	9.91	0.38	0.13	0.08	0.63
42	batch	sterile 20% DM+Fe	-	26	15.02	8.91	0.34	0.13	0.07	0.59
42	batch	sterile 20% DM+P	-	22	19.54	8.60	0.39	0.16	0.07	0.44

5 Polyhydroxyalkanoates and food safety

5.1 Application as food packaging materials

Plastics are used in many applications such as building blocks, toys, textiles, cosmetics including hygiene products, medical devices, sport products, packaging materials and many more (Koller, 2014; Reddy *et al.*, 2003). These broad applications are based on their mechanical and chemical properties as well as their lower cost compared to other materials like wood, natural fibers, and metals.

Among various applications of plastics, packaging materials account for 37% of the entire plastic market (Koller, 2014), food packaging included. Due to the changes in the market to more convenience products, the demand for packaging materials has equally increased. Progress in terms of industrialization, urbanization and globalization has led to changes in lifestyle and diets all over the world (Marina *et al.*, 2017). Many people live on convenience food, wrapped in plastic. Even small portions are packaged for hygienic reasons and for the purpose of convenience. However, packaging also serves the purpose of external shock protection during the whole food supply chain from production to distribution.

The problem is that most plastics are petroleum-based polymers, which have a negative impact on the environment. These plastics are not biodegradable and most of this plastic waste is disposed of by burial in landfills, by recycling, incineration and composting (Kale *et al.*, 2007). The more waste is disposed in landfills, the more the greenhouse effect is strengthened and the more diverse habitats are destroyed. Therefore, biodegradable products such as bioplastics have been developed as one of possibilities to replace petroleum-based polymers. Bioplastics are defined as plastics derived from renewable sources. Mostly they are produced and degraded in a chemical process by living organisms (Peelman *et al.*, 2013).

Bioplastics which have similar properties to petroleum-based plastics are thermoplasticized starch, polylactic acid (PLA), biobased polyethylene (PE), polytrimethylene (PTT), polybutylenes succinate (PBS), poly-p-phenylen (PPP) and microbial polyhydroxyalkanoates (PHAs) (Koller, 2014; Peelman *et al.*, 2013). Those biopolymers, with their certain mechanical and chemical properties, can be used in special fields like packaging, consumer goods, automobile parts, construction, agriculture and electronics etc. The PHAs have numerous properties due to their structural variations which lead to various applications. For example, PHAs show a low moisture permeability which is an advantage for food packaging materials, comparable to low-density polyethylene (LDPE). Poly-3-hydroxybutyrate (PHB)

which is one of the most common PHAs, has similar mechanical as well as thermal properties, melting temperature at 175–180 °C, to polypropylene (PP) (Liu, 2006).

Even though bioplastics are sustainable products whose life cycle is almost infinite, the commercialization of bioplastics as food packaging materials has still a long way to go due to several limitations.
The main limiting factor is the relatively expensive production cost of bioplastics (Peelman *et al.*, 2013). Therefore, the production process of PHB itself needs to be enhanced further in order to drive down costs in a substantial manner.
Another limitation is the slightly different properties of bioplastic. It has been reported for example that PHB is stiffer and more brittle than PP, hence, packaging made of PHB has poor impact resistance (Liu, 2006; Peelman *et al.*, 2013).
A further challenge faced by the food packaging industry is related to the compatibility with packaged food. Research has shown that during its shelf life sensory properties of different foods can be changed or impaired (Bucci *et al.*, 2007).

This chapter will provide an overview of polyhydroxyalkanoates (PHAs) including their properties, the improvement of these properties for their practical use as food packaging materials and an explanation about which bioplastics are currently commercially available.

5.2 Impact on food safety

To ensure the safety of food products, all possible (micro)biological, chemical or physical hazards should be eliminated or reduced to an acceptable level. The main aim of packaging is to guarantee food safety and quality, which signifies to protect food products from hazards occurring in the food chain. Besides this, packaging helps to trace back and forth, to transport efficiently and to minimize food waste (Manitoba, 2018).

To evaluate the possibilities of bioplastics as food packaging materials, the following properties are considered: firstly, to what extent materials can be protected against microbiological contamination, secondly the potential migration of components into food products as well as the release of toxic compounds from the used materials. Regarding potential quality issues, it will be analyzed to what extent packaging materials can resist external and internal impacts, namely temperature, pressure, gas, water or ultraviolet light. Lastly, the compatibility of PHB packaging with different food types undergoes further investigation.

5.2.1 Antimicrobial aspects

The food contaminated by microorganisms is not desirable to be consumed due to, not only its off-odor and off-taste but also due to the danger of a foodborne illness it might cause. Therefore, one of the criteria for food packaging is to protect the food against microbial contamination and to keep the number of microorganisms present in the food within the acceptable level (Adams and Moss, 2008).

The food wrapping with antimicrobial films is often used to enhance the shelf life and to avoid the contamination by foodborne pathogens, a method which ultimately improves food safety and quality. The films containing antimicrobial compounds belong to category called active food packaging materials which play an increasingly important role in food technology. Antimicrobial compounds are mostly used against foodborne pathogens and spoilage bacteria such as *Escherichia coli*, *Salmonella* species and *Staphylococcus aureus* as well as fungi such as *Aspergillus* species and *Penicillium* species. (Xavier *et al.*, 2015)

Natural compounds such as fatty acids and flavoring agents like vanillin can also be used as antimicrobial substances. Thus, these natural preservatives can contribute to prolonging the shelf life. Additionally, if these compounds are incorporated into bioplastics, these bioplastics are equipped with antimicrobial properties and are thus applicable for environmentally-friendly purposes. Xavier *et al.* (2015) reported that PHB films incorporated with eugenol and pediocin were effective to avoid food spoilage microorganisms. Moreover, PHB films associated with vanillin (4-hydroxy-3-methoxybenzaldehyde) also displayed an antimicrobial activity against bacteria and fungi, with more than 80 µg of vanillin per g PHB and 50 µg of vanillin per g PHB, respectively. The advantage of combining those antimicrobial compounds with PHB is that incorporation into the polymeric matrix advances their slow release, which might inhibit microbial growth for a longer time compared to the incorporation of the antimicrobial compounds into food matrices directly (Xavier *et al.*, 2015).

5.2.2 Chemical aspects

Chemical hazards encompass pesticides and other chemicals that might be applied to or present on raw materials, as well as chemicals used in the food manufacturing process including cleaners and sanitizers. Chemical hazards also include nonviable hazards such as proteins which can provoke allergic reactions (Batt, 2016). Packaging materials in contact with food products might release chemicals or can be the source of physical hazards. However, it is reported that PHAs as well as the hydrolyzed forms by PHA hydrolase and PHA depolymerase, R- and S-hydroxybutyrates, are non-toxic under aerobic and anaerobic conditions (Khosravi-Darani, 2015; Xavier *et al.*, 2015). Moreover, PHAs including PHB have a potential for medical applications (Hung *et al.*, 2015; Khosravi-Darani, 2015), which

indirectly proves that it is safe to be consumed. It has to be emphasized that the PHAs are extracted with toxic chemicals such as chloroform and sodium hypochlorite (Hahn *et al.*, 1995; Hassan *et al.*, 2013; Liu *et al.*, 2015). The potential migration of those chemicals into food, considering the potential impact on the human body needs to be further investigated for safety precautions.

Regarding the quality issue, oxidation, water loss and degradation of the food products should be considered. To avoid these, the barrier properties such as water vapor, gas permeability and UV light resistance have to be investigated, as they represent inevitable criteria for PHAs as food packaging materials.

It is reported that PLA-PHB blends, incorporating catechin which has a high antioxidant activity, are applicable for fatty food as the release of catechin inhibits the lipid oxidation (Arrieta *et al.*, 2014a).

It has also been shown that PLA-PHB blends coated with surfactant modified cellulose nanocrystals (CNCs) enhanced water resistance, reduced oxygen and UV-light transmission and might thus be suitable as packaging materials. The better water resistance can be explained as the presence of sulfate groups with low polarity increases the hydrophobicity on the surface of the product (Arrieta *et al.*, 2014b). The active packaging films made of PLA-PHB blends combining with D-limonene, a natural terpene, were more transparent and flexible than without natural terpene. Furthermore, these blends showed an increase of the oxygen barrier and the water resistance (Xavier *et al.*, 2015).

Biopolymers combining with active compounds open more prospects due to their enhanced properties. However, taking into account safety and environmental issues, natural preservatives or substances from renewable resources are recommended for the usage of active compounds instead of synthetic additives (Arrieta *et al.*, 2014a).

5.2.3 Mechanical/thermal aspects

To protect food products from external shocks and vibrations during storage and transportation etc., impact resistance and thermal stability of packaging materials should be investigated. Thus parameters such as tensile strength, elongation degree at break, melting temperature and temperature for degradation have to evaluated (Giaquinto *et al.*, 2017).

There are various properties exhibited by PHAs depending on their side chains. For example, scl-PHA is brittle and has a poor elastic property, while mcl-PHA is moldable. However, PHB, which is a scl-PHA, was the first polymer discovered among the PHAs and has similar properties in comparison with petroleum-derived plastics. Many studies have researched the

PHB as a potential packaging material. It has been shown that the deformation value of PHB was lower than that of polypropylene (PP) due to the higher rigidness (Bucci *et al.*, 2005).

Long storage at room temperature of PHB causes the increase of its brittleness; therefore PHB can be suggested for the use of short term food packaging materials (Xavier *et al.*, 2015). However, the PHB blended with other polymers can improve this mechanical property. For instance, PHB coated with an acetylated cellulose film has resulted in higher elasticity and higher tensile strength (Peelman *et al.*, 2013).

PLA can be processed with PHB due to their similar melting temperatures and their processability. Additionally, PLA-PHB blends can be improved by the addition of plasticizers such as ATBC (acetyl-tributyl citrate), which is regarded as a safe compound for materials in contact with food, as they are more flexible. PLA-PHB plasticized with ATBC showed a higher elongation value at break than other plasticizers like polyethylene glycol (PEG) and D-limonene (Arrieta *et al.*, 2014a).

The combination of PLA-PHB blends with surfactant modified cellulose nanocrystals (CNCs) also improved the tensile strength and increased the elongation at break. This might be explained with the enhanced interfacial adhesion caused by functionalized cellulose nanocrystals contributing to a better interaction between polymers. Furthermore it leads to an improvement of the thermal stability of both polymers (Arrieta *et al.*, 2014b).

Even though PHB has a relatively high melting temperature and a high degree of crystallinity (Bucci et al., 2005), it thermally decomposes at a temperature close to its melting point. It has been observed that a short exposure of PHB to temperatures close to 180 °C induced a severe degradation of olefinic and carboxylic acid compounds etc. (Bugnicourt *et al.*, 2014). It might thus be a problem to process PHB due to the low resistance to thermal degradation. However, the addition of plasticizers can modify the thermal properties (Bugnicourt *et al.*, 2014). In other words, polymers with plasticizers can decrease the processing temperature or increase its melting temperature. Arrieta *et al.* (2014a) reported that PLA-PHB blends incorporated with catechin increased thermal stability.

5.2.4 The compatibility of PHB with different types of food products

There has been increased effort to use bioplastics including PHB as packaging materials. Regarding the shelf life of food products, bio-derived plastics are supposed to be stable without any changes in mechanical, chemical and sensorial properties until the package is disposed of. The compatibility of these bioplastics with food products also must be considered to ensure food safety and quality.

As mentioned above, PHB has a better UV light barrier than polypropylene (PP), suggesting this biopolymer for the packaging of milk or oil products which need a good UV light resistance (Bucci *et al.*, 2007). Moreover, it was observed in this study that no migration of components from PHB packaging of bottles has occurred for fat rich foods (Bucci *et al.*, 2007). It has also been reported that polypropylene (PP) films could be replaced by PHB films for fat rich foods such as mayonnaise, margarine, cream cheese and sour cream (Peelman *et al.*, 2013; Xavier *et al.*, 2015).

Conventional plastics such as polyethylene (PE) and polypropylene (PP) as well as biopolymers like PLA and PHB used for meat packaging, combined with the sous vide method, were also investigated. It was found that the PLA and the PHB films could also be utilized, since these films proved to be resistant against heat treatment up to 65 °C and what is more, also showed a similar antimicrobial property, compared to the PE and the PP films. (Levkane *et al.*, 2008)

It was reported that the quality of artificially flavored orange juice and dressing packed with PHB was comparable to that of high-density polyethylene (HDPE). Thus, commercial juices and other acidic beverages as well as dressings can possibly be packed with PHB (Haugaard *et al.*, 2002).

Further research focused on the issue of sensorial perception of food products packed with PHB. It was found that the PHB packaging material is not recommended for aqueous products, because the water packed in PHB bottles showed a remarkable difference in the organoleptic test. In contrast, the fat rich products such as mayonnaise, margarine and cream cheese showed no noticeable difference of sensorial deterioration, even though those products are more susceptible to changes in sensorial perception than other food products (Bucci *et al.*, 2005).

Catechin incorporated polymers showed an effective release of catechin during the first 10 days into food products, signifying that oxidation of food could be inhibited up to 10 days by the antioxidant catechin (Arrieta *et al.,* 2014a). It has also been observed that flavoring agents and natural preservatives with antimicrobial activity inhibited the contamination of foodborne pathogens as well as the growth of mold. This indicates that PHB combined with those substances is suitable for the packaging of freshly cut fruit and vegetables as well as bread.

5.3 Market

PHAs are readily available on the market. Table 21 (Bugnicourt *et al.*, 2014) summarizes the commercially available PHAs plus the countries of production.

PHAs based natural raw materials are processed for the cosmetics as well as the pharmaceutical sector in Germany and Italy. One German company, called Biomer uses renewable resources for the production of PHB with no toxicity detected up to now (Biomer, 2018). Minerv SC and SB are PHAs produced by Bio-on, an Italian company. They use the fermentation process with renewable resources like sugar beet and sugar cane waste as carbon sources. It is proven that those PHAs granules are applicable for injection and extrusion to produce films or bottles (Bio-on, 2018). Goodfellow Cambridge Ltd. in the UK manufactures PHB and its copolymer, PHB-HV, as well (Bugnicourt *et al.*, 2014).

In the USA, there are also some companies providing PHB for medical devices and food packaging materials; e.g. Biopol (Metabolix) (Liu, 2006), P&G Chemicals (Procter and Gamble) (da Silva Pinto *et al.*, 2009) and Tepha Inc. etc. In the 1980s, Imperial Chemical Industries developed poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fermentation, named Biopol. Biopol now belongs to Metabolix, since they acquired its patent. Biopol products are made with carbon from natural resources like sugar, starch, vegetable oils, and cellulose instead of fossil carbon. P&G Chemicals manufactures P(HB-coHHx) with a wide range of customizable mechanical properties from soft/elastic to hard (Liu, 2006; Metabolix, 2018).

Biocycle is produced by PHB Industrial in Brazil. The development of Biocycle started in 1992, focusing on the production of biodegradable polymers. They then developed a technology to extract and purify bioplastics, using a superior alcohol as a solvent. In 1995, the pilot plant was constructed and the production of PHB and PHB-HV, deriving from natural sugar cane, was started. Their molecular weight varies from 150,000 to 600,000 Da. The capacity of the pilot plant was 5 tons/year, increasing to 50 tons/year in 2000. (Biocycle, 2018)

PHAs are also manufactured in Asia. The main manufacturers are Tianan Biological Materials Co. Ltd. and Jiangsu Nantian Group in China and Mitsubishi Gas in Japan. Tianan Biological Materials Co. Ltd. had a production capacity of 2,000 tons/year in 2000 and increased their capacity to 10,000 tons/year in 2009. This company uses dextrose derived from corn or cassava to produce PHB with *Cupriavidus necator* (*Ralstonia eutropha*). Since they cooperate with the Dutch firm DSM, they have expanded the marketing of PHB to Europe. (TianAn Biologic Materials Co. Ltd., 2012)

The price of biopolymer ranges from 1.6 to 15 €/kg, as can be seen in Figure 13 (Endres and Siebert-Raths, 2011). The PHAs price has a wide range from 3 to 15 €/kg, whereas other biopolymers cost 3 to 6 €/kg. Moreover, bacterial plastics cost 5 to 10 times more, in comparison with the price of petroleum-based plastics, which is below 1 U.S. dollar per kg (Scheller and Conrad, 2005). PHAs are available with a higher price, because of the higher material costs as well as a relatively small scale of production by the manufacturer, compared to the other biopolymers as well as the synthetic polymers. To increase the availability of PHAs for food packaging materials, the price has to be reasonably reduced. Considering the economic aspect, the price of PHAs will most probably decrease in the upcoming years as the capacity of production has been increasing and as there has been much research of PHAs production with cheaper raw materials such as by-products from the sugar manufacturing companies and waste from oil production etc. Not only the low-price of raw materials, but also the further development of efficient extraction and purification methods will contribute to reduce its price (Scheller and Conrad, 2005).

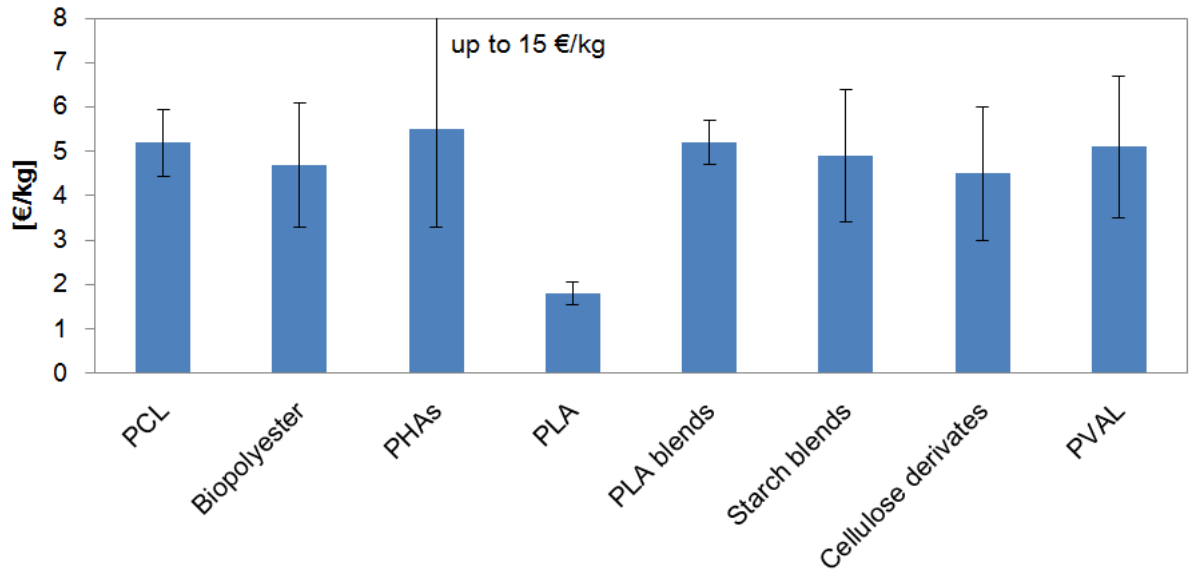


Figure 13: Price of various biopolymers (Endres and Siebert-Raths, 2011).

Table 21: Commercial PHAs: producer, name, product and type of biopolymer (data adapted from Bugnicourt *et al.*, 2014).

Producer	Country	Commercial name	Product	Type of biopolymer
Biomer	Germany	Biomer	Biomer P209, P226, P240	PHB
Bio-on	Italy	Minerv-PHA	MINERV-PHA SB (sugar beet), SC (sugar cane)	PHAs
Biopol (Metabolix)	USA	Mirel	Mirel P4001, P4010, P5001, P5004, M2100, M2200	P(3HB-co-3HV)
Goodfellow Cambridge Ltd.	UK	Goodfellow		PHB, PHB/PHV-88%/12%, PHB/PHB-92%/8%
Jiangsu Nantian Group	China	Jiangsu Nantian		P(3HB)
Mitsubishi Gas	Japan	Biogreen	Biogreen	PHB
P&G Chemicals (Procter and Gamble)	USA/Japan	Nodax	Nodax™	P(HB-coHHx)
PHB Industrial	Brazil	Biocycle	Biocycle 1000, 18BC-1, 189C-1, 189D-1	PHB, PHB-HV
Tepha Inc.	USA	Tepha FLEX		P(4HB)
Tianan Biological Materials Co. Ltd.	China	Ecogen	Enmat Y1000, Y1000P, Y1010, Y3000P	PHB, P(3HB-co-3HV)

5.4 Regulations

Food packaging materials are directly or indirectly in contact with food products; thus, they might have an impact on them. Due to this, there are regulations regarding FCMs (Food Contact Materials) to ensure food safety.

The materials themselves as well as additional substances to improve their properties have to be controlled and have to comply with the EU regulation 1935/2004. The EU regulation No 10/2011, however, states a specific measure for plastic materials and articles intended to come in contact with food. Especially, for plastics the EU regulation 2002/72/EU should be considered. In the USA, the U.S. Food and Drug Administration set the requirements regarding direct and indirect food contact materials in the Code of Federal Regulations, Title 21 (U.S. FDA 2017). To comply with these regulations, the manufacturers deliver appropriate compliance documentation like safety data sheets and food contact statements.

Not only the regulations, but also several standards have been developed to support the safe and hygienic manufacturing of packaging materials; for example, the ISO EN 15593, the ISO 22000 and the BRC IOP Global Standard for Packaging and Packaging Materials.

Apart from the food contact materials, the safety of materials in general should be observed based on (EG) No. 1907/2006 to protect human health and the environment.

5.5 Future prospects

Based on the research so far, PHB properties and their non-toxicity make them suitable for food packaging materials. The cost will most probably decrease eventually, as the demand of PHAs has increased and as there are more and more companies involved in PHAs production. To make the usage of PHAs more readily available, the product quality of PHAs also should be improved. More specifically, the processing strategies such as the injection or extrusion parameters for the final product (e.g. film or bottles etc.), have to be optimized for PHAs. Finally, the interaction between foods and those final goods needs to be further investigated.

6 Conclusions

In this study, various factors, including the media conditions, the fermentation parameters and the feeding strategies, have been investigated to optimize the process of PHB production by *Bacillus megaterium* CECT 7922.

The findings of this study can be summarized as follows:

- A similar growth behavior of *Bacillus megaterium* CECT 7922 was observed in the desugarized molasses from the batches of 2016 and 2017. The 2017 batch, however, led to a higher PHB yield with the same concentration of desugarized molasses as the 2016 batch. This finding is based on the different chemical compositions of each batch. It is advisable that desugarized molasses from different batches are diluted accordingly so that sugar concentration in all media is comparable. It helps to determine an optimum desugarized molasses concentration with each batch for biomass and PHB production.
- A faster and a better growth rate of *Bacillus megaterium* CECT 7922 as well as a higher PHB concentration were achieved, when KCl instead of NaCl was added to the medium. Even high concentration of KCl (1.71M) did not inhibit the growth of *Bacillus megaterium* CECT 7922.
- Adding betaine and iron into the desugarized molasses did not result in any increase of CDW and PHB production. On the other hand, a higher concentration of biomass as well as a faster growth rate was noticed, when phosphate was added to the medium.
- The optimum pH value for the PHB production by *Bacillus megaterium* CECT 7922 is between 7 and 8. Less CDW and PHB were produced in the medium with pH 8.2. With pH values below pH 6 or above pH 8.5 no growth was noticed.
- Compared to the batch cultivation, the fed-batch fermentation almost doubled the CDW as well as the PHB concentrations. The highest concentrations of CDW and PHB were obtained, when 80% desugarized molasses was used as a feed.
- The use of unsterile desugarized molasses resulted in no growth of *Bacillus megaterium* CECT 7922. When this unsterile desugarized molasses was fed to a strongly growing batch, containing the sterile desugarized molasses however, *Bacillus megaterium* CECT 7922 continued growing. Nevertheless, the PHB productivity as well as the yields of CDW and PHB were lower than the data obtained in the sterile fed-batch fermentation.

- Considering the cultivation time, the PHB productivity was higher in the batch process, in comparison with the fed-batch fermentation. In addition to this, slightly higher yields of CDW and PHB as well as a higher PHB content of CDW were achieved.
- Literature research confirmed the potential use of PHB as a food packaging material. This assumption is based on the observation that PHB and its copolymers as well as PHB incorporated with natural compounds showed a good antimicrobial activity, gas/water/UV resistance, thermal stability as well as tensile strength thus ensuring adequate food safety and quality. Furthermore, literature research showed that PHB is as compatible with fat-rich foods and meat products as conventional plastics.

It can be stated that *Bacillus megaterium* CECT 7922 is a promising PHB producer, since it managed to produce PHB in all kinds of media used in this study. Only in the case of media contaminated by other microorganisms, the growth of *Bacillus megaterium* CECT 7922 was substantially inhibited.

For large scale PHB production, preference should be given to the batch cultivation as opposed to the fed-batch process. A pH range from 7 to 8 is recommended. To enhance the biomass and the PHB production, potassium or phosphate could be added to the medium.

Further research is suggested regarding the enhancement of PHB production using unsterile desugarized molasses to reduce PHB production costs. Moreover, it might be beneficial to develop an efficient downstream process, in terms of extraction and purification of the obtained PHB.

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Annex

Table A1: pH values with different concentrations of desugarized molasses (IFA Nr.47).

Duration	15%	17%	20%	25%
0:00	7.11 ± 0.01	6.89 ± 0.00	6.87 ± 0.00	6.92 ± 0.00
2:30	7.08 ± 0.02	6.91 ± 0.01	6.89 ± 0.00	6.94 ± 0.00
5:00	7.07 ± 0.01	6.91 ± 0.00	6.88 ± 0.00	6.93 ± 0.00
21:00	7.01 ± 0.05	7.08 ± 0.04	6.98 ± 0.21	6.88 ± 0.01
24:00	7.17 ± 0.03	6.80 ± 0.16	7.02 ± 0.23	6.86 ± 0.01
26:30	7.22 ± 0.24	6.65 ± 0.30	7.13 ± 0.18	6.82 ± 0.01
28:30	- -	7.00 ± 0.41	6.92 ± 0.20	6.78 ± 0.00
45:00	- -	8.42 ± 0.05	7.33 ± 0.49	6.54 ± 0.05
48:00	- -	8.64 ± 0.21	7.64 ± 0.64	6.61 ± 0.10
51:30	- -	8.81 ± 0.34	7.97 ± 0.35	6.96 ± 0.26
57:00	8.62 ± 0.51	- -	- -	- -
69:00	- -	9.07 ± 0.47	8.84 ± 0.48	7.62 ± 0.77
72:00	- -	9.12 ± 0.44	8.90 ± 0.50	7.88 ± 0.76
75:30	- -	9.26 ± 0.27	8.94 ± 0.50	8.16 ± 0.43
93:00	- -	9.64 ± 0.01	9.37 ± 0.20	8.60 ± 0.19

Duration	30%	35%	40%
0:00	7.01 ± 0.00	6.98 ± 0.00	6.99 ± 0.00
2:30	6.98 ± 0.01	6.97 ± 0.00	6.99 ± 0.00
5:00	6.99 ± 0.01	6.97 ± 0.01	6.97 ± 0.00
21:00	6.94 ± 0.00	6.91 ± 0.00	6.92 ± 0.00
24:00	6.93 ± 0.00	6.90 ± 0.00	6.90 ± 0.00
26:30	6.92 ± 0.00	6.89 ± 0.00	6.89 ± 0.00
28:30	6.89 ± 0.00	6.86 ± 0.01	6.86 ± 0.01
45:00	±	±	±
48:00	6.88 ± 0.00	6.85 ± 0.00	6.85 ± 0.00
51:30	6.86 ± 0.00	6.83 ± 0.00	6.83 ± 0.01
57:00	- -	- -	-
69:00	6.88 ± 0.07	6.83 ± 0.00	6.83 ± 0.00
72:00	6.88 ± 0.05	6.82 ± 0.00	6.82 ± 0.00
75:30	6.64 ± 0.35	6.80 ± 0.00	6.86 ± 0.06
93:00	5.82 ± 0.11	6.80 ± 0.00	6.80 ± 0.00

Table A2: Additional data (the average and error range) from Figure 7 (IFA Nr.91 and 98); Unit for CDW and PHB is g/L.

		KCl								NaCl							
Duration		**OD600**	**±**	**pH**	**±**	**CDW**	**±**	**PHB**	**±**	**OD600**	**±**	**pH**	**±**	**CDW**	**±**	**PHB**	**±**
0:00	0.086M	0.12	0.00	6.84	0.00					6.84	0.00	0.10	0.00				
3:00		0.42	0.01	6.73	0.01					6.73	0.01	0.35	0.01				
6:00		7.66	0.26	5.44	0.00					5.71	0.06	6.16	1.14				
24:00		29.38	0.77	6.42	0.04	9.23	0.07	5.59	0.04	5.63	0.33	18.98	1.63	5.90	0.30	2.32	0.30
30:00		28.53	0.22	6.74	0.04					6.56	0.03	20.15	0.30				
48:00		26.70	0.95	6.10	0.18	8.47	0.11	5.29	0.09	6.32	0.18	20.53	1.38	6.40	0.28	2.68	0.08
72:00		24.10	0.25	6.79	0.15					6.96	0.08	17.85	0.25				
0:00	0.17M	0.12	0.00	6.79	0.00					6.77	0.00	0.11	0.00				
3:00		0.39	0.00	6.71	0.00					6.69	0.00	0.32	0.00				
6:00		6.14	0.14	5.59	0.05					5.94	0.01	3.62	0.04				
24:00		25.28	0.38	6.04	0.09	8.3	0.08	4.89	0.02	5.75	0.11	22.70	1.10	6.97	0.23	3.38	0.26
30:00		25.73	0.13	6.58	0.02					6.65	0.03	22.40	0.45				
48:00		23.23	1.03	6.15	0.03	7.51	0.13	4.48	0.00	6.14	0.28	21.43	2.83	6.51	0.09	3.16	0.15
72:00		23.03	0.52	6.54	0.04					6.59	0.08	18.10	0.00				
0:00	0.43M	0.12	0.00	6.70	0.01					6.63	0.00	0.12	0.00				
3:00		0.31	0.01	6.66	0.00					6.60	0.00	0.22	0.00				
6:00		1.68	0.27	6.34	0.02					6.22	0.03	2.02	0.12				
24:00		22.45	2.70	5.71	0.29	7.02	0.76	3.83	0.73	5.06	0.31	21.23	1.88	6.16	0.40	2.76	0.31
30:00		24.20	0.50	6.64	0.07					6.58	0.04	21.58	0.57				
48:00		22.28	1.68	6.40	0.02	6.73	0.25	3.74	0.00	6.04	0.37	18.68	0.07	5.78	0.06	2.67	0.01
72:00		18.98	0.77	6.05	0.00					6.28	0.05	17.45	0.75				

Table A2: Additional data (the average and error range) from Figure 7(IFA Nr.91 and 98) (continued); Unit for CDW and PHB is g/L.

		KCl								NaCl							
Duration		**OD600**	**±**	**pH**	**±**	**CDW**	**±**	**PHB**	**±**	**OD600**	**±**	**pH**	**±**	**CDW**	**±**	**PHB**	**±**
0:00	0.86M	0.13	0.13	6.86	0.01					6.47	0.00	0.13	0.00				
3:00		0.20	0.20	6.84	0.01					6.81	0.07	0.11	0.00				
6:00		0.62	0.64	6.67	0.00					6.80	0.07	0.14	0.00				
24:00		12.45	13.20	4.49	0.20	4.36	0.28	1.92	0.07	4.65	0.02	15.03	0.98	4.25	0.53	1.38	0.23
30:00		15.80	17.15	6.35	0.02					6.43	0.02	15.58	0.48				
48:00		20.90	18.80	6.58	0.07	5.84	0.08	2.88	0.04	6.11	0.11	16.18	0.02	4.74	0.10	1.67	0.10
72:00		17.30	16.90	5.98	0.02					6.10	0.04	14.03	0.92				
0:00	1.28M	0.13	0.00	6.88	0.00					6.34	0.00	0.13	0.00				
3:00		0.14	0.00	6.86	0.00					6.90	0.01	0.12	0.00				
6:00		0.19	0.00	6.84	0.00					6.89	0.02	0.13	0.00				
24:00		12.60	0.90	5.03	0.04	4.08	0.46	1.34	0.23	5.99	0.04	4.83	0.39	3.76	0.00	0.24	0.00
30:00		15.25	1.00	6.37	0.05					5.97	0.03	8.67	0.59				
48:00		15.30	0.05	5.86	0.07	4.89	0.13	1.92	0.05	4.85	0.13	15.58	1.63	4.58	0.02	1.44	0.13
72:00		15.00	0.80	5.80	0.07					5.93	0.04	14.63	0.27				
0:00	1.71M	0.14	0.00	6.82	0.01					6.22	0.00	0.13	0.00				
3:00		0.19	0.06	6.82	0.01					6.84	0.01	0.12	0.00				
6:00		0.13	0.00	6.82	0.00					6.83	0.02	0.12	0.00				
24:00		1.33	0.23	6.53	0.03	1.25	0.17	0.00	0.00	6.82	0.02	0.11	0.00				
30:00		2.15	0.51	6.79	0.11					6.80	0.00	0.11	0.00				
48:00		12.08	0.63	4.91	0.46	4.27	0.33	1.18	0.00	6.71	0.04	0.24	0.07	0.70	0.39	0.00	0.00
72:00		19.15	0.65	5.49	0.40	5.65	0.29	2.74	0.37	6.05	0.15	2.00	0.66				
144:00		20.70	3.35	6.03	0.02					4.99	1.24	8.42	5.44				

TableA3: Data of experiment with additional iron (Fe); [1)]: contains 0.00004 g/L of Fe (0.0002 g/L of $FeSO_4 \cdot 7H_2O$); [2)]: contains 0.009 g/L of Fe (0.025 g/ of $FeSO_4$); The data shown was obtained from the previous experiments carried out at IFA Tulln.

Phosphorus limitation [1)]					
Sample	Duration	OD600	CDW [g/L]	PHB [g/L]	PHB content [g PHB/g CDW]
1	0:00	0.21	-	-	-
2	7:00	3.31	1.22	0.10	8.53
3	22:00	15.48	6.28	2.18	34.69
4	24:00	15.43	6.60	2.28	34.59
5	26:00	15.15	6.88	2.23	32.43
6	28:00	17.55	7.02	2.31	32.89
7	30:00	17.20	7.34	2.40	32.73
8	47:00	18.68	7.22	2.07	28.66
9	49:00	18.98	7.18	2.04	28.47
10	51:00	18.13	7.38	1.93	26.10

Phosphorus limitation with additional Fe^{2+} [2)]					
Sample	Duration	OD600	CDW [g/L]	PHB [g/L]	PHB content [g PHB/g CDW]
1	0:00	0.75	-	-	-
2	7:30	25.15	5.18	1.80	34.74
3	22:30	32.30	7.01	3.47	49.52
4	25:30	30.95	6.59	3.44	52.19
5	27:30	29.65	6.07	3.12	51.34
6	29:30	29.60	6.17	3.31	53.62
7	46:30	26.95	5.70	2.32	40.62
8	48:30	26.98	5.55	2.21	39.88
9	51:30	32.65	6.37	3.14	49.25

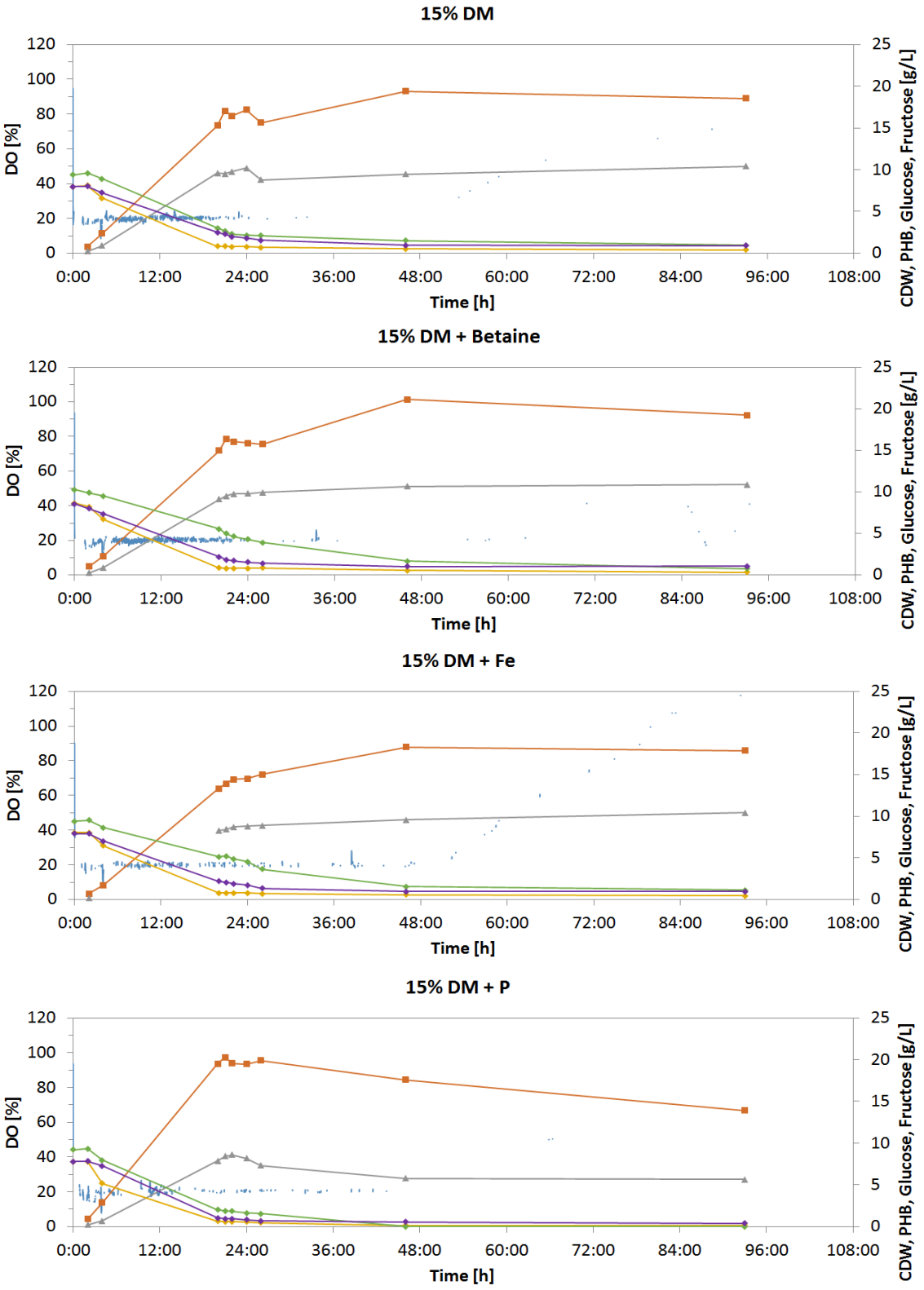

Figure A1: The graphical data of the experiment (bioreactor cultivation) with 15% DM and the additional substances (IFA Nr.42).

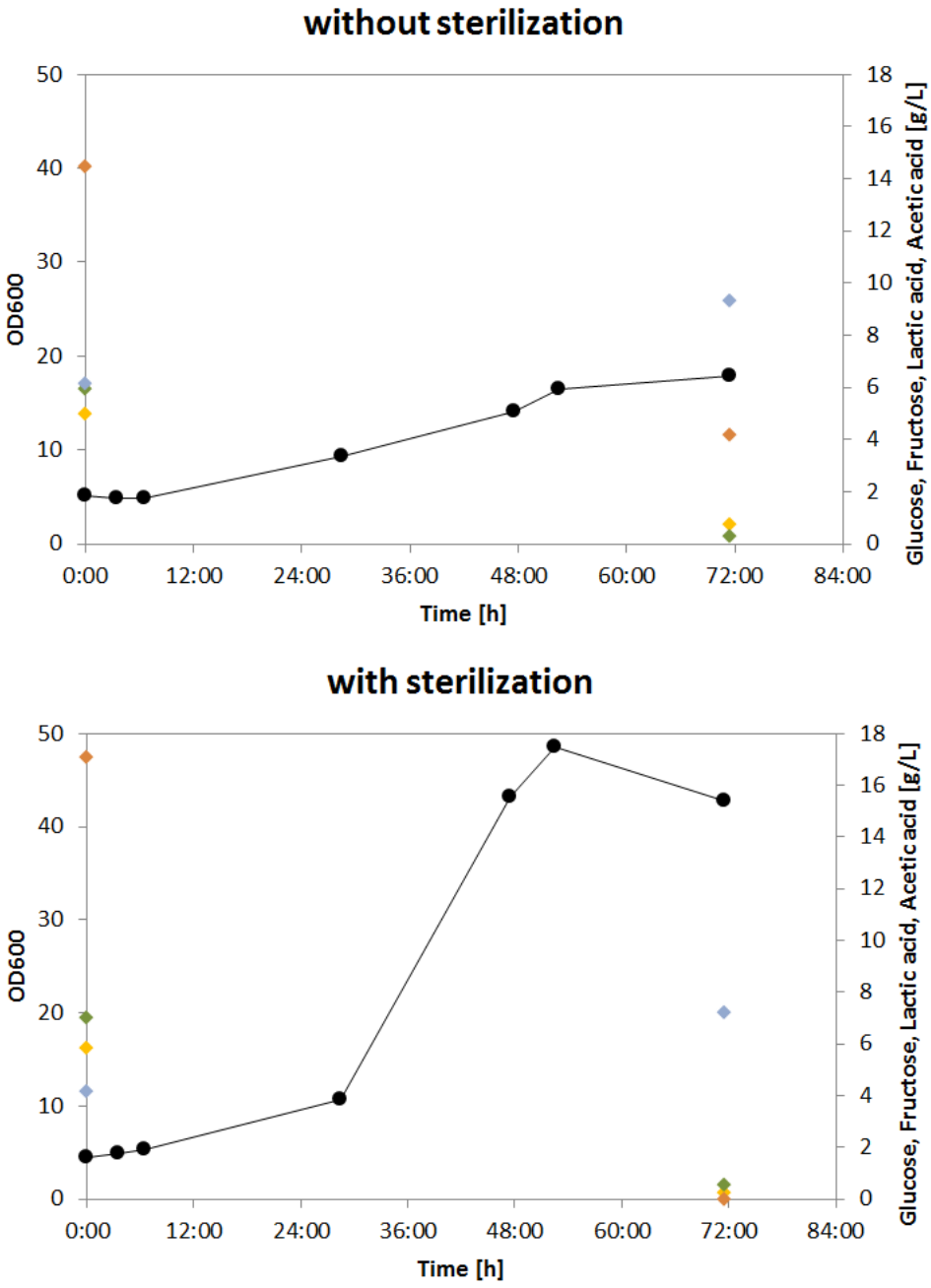

Figure A2: Graphical data of the experiment with the unsterile and the sterile desugarized molasses (IFA Nr.63); Four flasks with the desugarized molasses without any treatment were prepared; This graph shows only the average value; The average and error range of the data from four different flasks are present in the Annex Table A4.

Table A4: Additional data (the average and error range) from Figure A2.

	Without sterilization											
Dura -tion	**OD600**		**pH**		**Glucose [g/L]**		**Fructose [g/L]**		**Lactic acid [g/L]**		**Acetic acid [g/L]**	
0:00	5.09	+0.36	6.98	+0.00	13.84	+1.25	16.55	+1.52	14.48	+1.36	6.12	+7.64
		-0.54		-0.02		-1.77		-2.15		-1.89		-3.03
3:30	4.84	+0.06	7.06	+0.03								
		-0.04		-0.04								
6:30	4.80	+0.20	7.06	+0.01								
		-0.15		-0.02								
28:30	9.35	+1.10	6.83	+0.31								
		-0.95		-0.81								
47:30	14.05	+4.95	7.29	+0.75								
		-3.20		-1.92								
52:30	16.46	+6.79	7.21	+1.03								
		-4.31		-1.85								
71:30	17.94	+4.16	7.36	+1.25	2.08	+5.23	0.84	+0.26	4.15	+7.14	9.33	+4.91
		-6.74		-1.98		-1.77		-0.14		-2.91		-6.51

	With sterilization					
Dura -tion	**OD600**	**pH**	**Glucose [g/L]**	**Fructose [g/L]**	**Lactic acid [g/L]**	**Acetic acid [g/L]**
0:00	4.45	7.13	16.22	19.41	17.06	4.16
3:30	4.85	7.11				
6:30	5.30	7.12				
28:30	10.65	7.16				
47:30	43.25	7.52				
52:30	48.50	8.22				
71:30	42.75	8.82	0.64	1.46	0.00	7.19

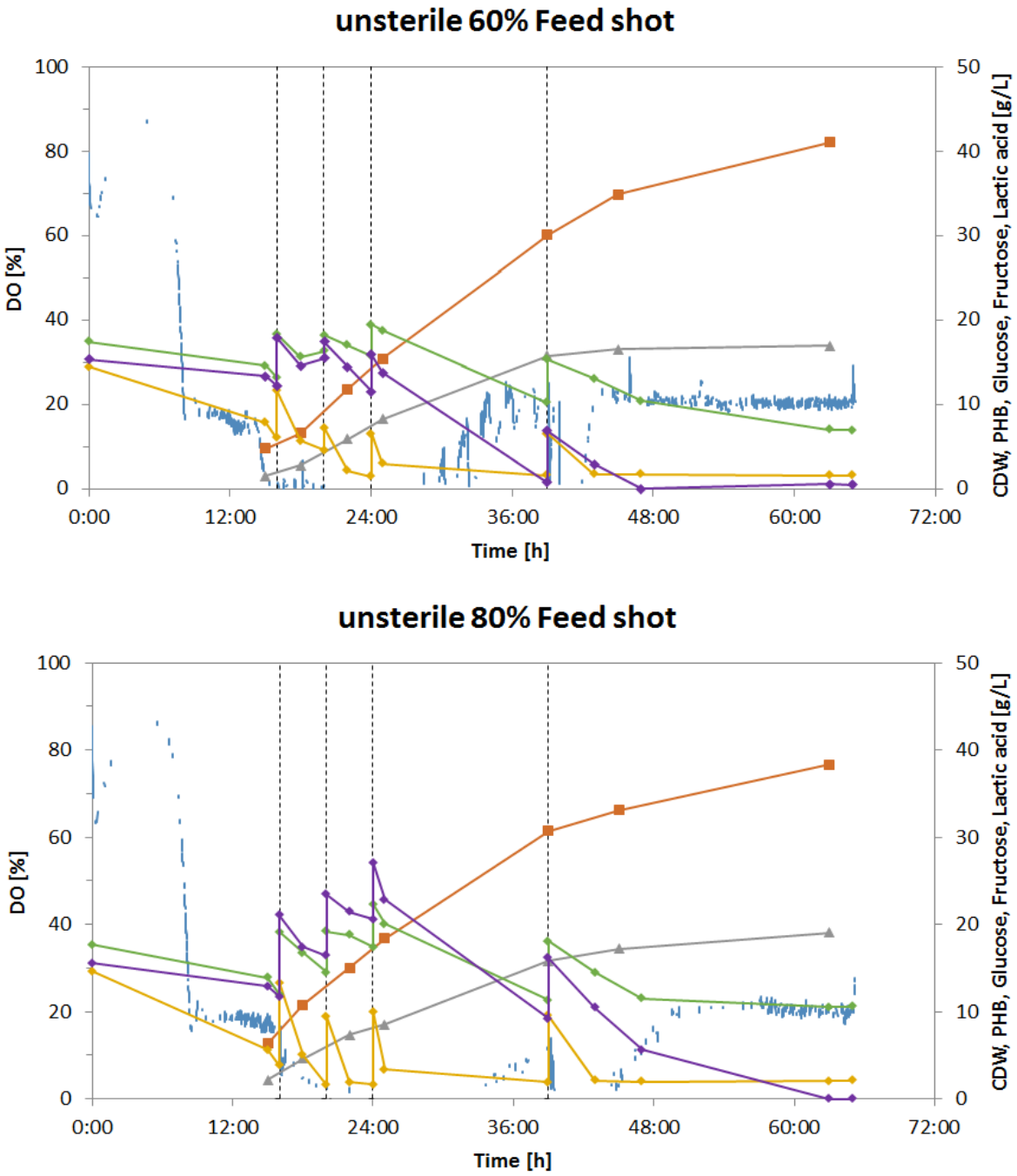

Figure A3: Graphical data of the experiment with the different unsterile feed concentrations (IFA Nr.94); The experiments were performed in duplicate. This graph shows only the average values.