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Cristiana Carvalho Morais

Degree in Marine Biology and Biotechnology

Production of bacterial biopolymers from industrial fat-containing wastes

Dissertation presented in partial fulfilment of the Requirements for the Degree of Master in Biotechnology

Supervisor: Doctor Maria Filomena Andrade de Freitas, Post Doctoral Researcher, REQUIMTE, FCT/UNL

Co-supervisor: Professor Maria da Ascensão Carvalho Miranda Reis, Full Professor, Faculdade de Ciências e Tecnologia, UNL

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Abstract

Polyhydroxyalkanoates (PHAs) constitute a group of biobased and biodegradable polymers, which have been recognized as good substitutes for petroleum-based polymers in many applications. The large-scale production of PHAs is limited by the high cost of the most commonly used carbon sources (e.g. glucose, sucrose). However, the food industry generates large amounts of wastes, including fat-containing materials that can be used as low cost carbon sources for microbial cultivation, due their high carbon content.

In this study, several bacterial strains (*Cupriavidus necator*, *Comamonas testosteroni*, *Pseudomonas oleovorans, P. resinovorans, P. stutzeri,* and *P. citronellolis*) were evaluated for their ability to grow and produce PHAs using fat-containing wastes generated by the food industry. The materials used in this study were mainly composed of free fatty acids, namely mystiric, oleic, linoleic and stearic acid. In the preliminary shake flask experiments, *C. necator*, *C. testosteroni*, *P. oleovorans* and *P. citronellolis* were able to grow and produce PHA polymer on margarine waste with the highest content. Those strains were selected for batch bioreactor experiments, wherein *C. necator* reached the highest polymer content (56%, wt/wt) and volumetric productivity (0.33 g_{PHA}/L.h), Lower PHA contents were achieved by *P. citronellolis* and *P. oleovorans* (7.0 and 8.5%, wt/wt, respectively). However, in contrast with *C. necator* that synthesized polyhydroxybutyrate [P3(HB)], those strains produced medium chain length polyesters (mcl-PHA) containing monomers of 3-hydroxyoctanoate (HO) and 3-hydroxydecanoate (HD). *C. necator* was also cultivated in two different fed-batch strategies. The first cultivation achieved 76% (wt/wt) of P(3HB), while high cell densities were obtained in the second cultivation (48 g/L of active biomass concentration). Finally, the P(3HB) and mcl-PHA polymers had a glass transition temperature of 0.5–7.9ºC and -45.6, a melting point of 169.3–173.4ºC and 60.9ºC, and degree of crystallinity of 48.7–56.6% and 0.7%, respectively.

Keywords*:* Fatty acids, waste, poly(3-hydroxybutyrate), medium-chainlength polyhydroxyalkanoates (mcl-PHAs), *Cupriavidus necator*, *Pseudomonas* species

Resumo

Os polihidroxialcanoatos (PHAs) são polímeros biológicos e biodegradáveis que são reconhecidos como bons substitutos dos polímeros produzidos à base de petróleo em muitas aplicações. Contudo, a produção em larga escala dos PHAs é limitada pelo elevado custo de muitas fontes de carbono utilizadas (ex. glucose, sacarose). A indústria alimentar gera enormes quantidades de resíduos, incluindo material gorduroso, que podem ser usados como fonte de carbono de baixo custo no cultivo microbiano devido ao seu elevado conteúdo em carbono.

Neste estudo, várias estirpes bacterianas (*Cupriavidus necator*, *Comamonas testosteroni*, *Pseudomonas oleovorans, P. resinovorans, P. stutzeri,* and *P. citronellolis*) foram avaliadas quanto à sua capacidade de crescer e produzir PHAs, usando resíduos de gordura gerados pela indústria alimentar. Estes resíduos eram compostos principalmente de ácidos gordos livres, nomeadamente, ácido místirico, oleico, linoleico e esteárico. Nos ensaios preliminares em frascos agitados, *C. necator*, *C. testosteroni*, *P. oleovorans* and *P. citronellolis* foram capazes de crescer e acumular polímero de PHA com o maior conteúdo, usando resíduos de margarina. Essas culturas foram cultivadas em reactor descontínuo, onde *C. necator* obteve o maior conteúdo de polímero (56%, m/m) e produtividade (0.33 g_{PHA}/L.h). Os conteúdos de polímero de *P. citronellolis* and *P. oleovorans* foram inferiores (7.0 e 8.5%, m/m, respectivamente). No entanto, em contraste com o poli(3-hidroxibutirato) [P(3HB)] produzido pela *C. necator*, estas estirpes produziram poliésteres de cadeia média, contendo monómeros de 3-hidroxioctanoato (HO) e 3-hidroxidecanoato (HD). Duas estratégias em reactor semi-contínuo foram aplicadas com *C. necator*. O primeiro sistema semi-contínuo obteve 76% (m/m) de conteúdo em polímero, enquanto no segundo sistema, elevada densidade celular foi obtida (48 g/L de biomassa activa). Os polímeros de P(3HB) e mcl-PHA alcançaram uma temperatura de transição vítrea de 0.5–7.9ºC e -45.6, um ponto de fusão de 169.3–173.4ºC e 60.9ºC, e uma cristalinidade de 48.7–56.6% e 0.7%, respectivamente.

Palavras-chave: Ácidos gordos, poli(3-hidroxibutirato), polihidroxialcanoatos de cadeia média (mcl-PHAs), *Cupriavidus necator*, *Pseudomonas*

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Nomenclature

[PHA] – Polymer concentration (g/L)

[Residual margarine waste] – Margarine waste concentration (g/L)

CDW – Cell dry weight (g/L)

OD600nm – Optical density at 600 nm

ppm - Part per million

q_{PHA} – Specific productivity (g_{PHA}/g_{X}.h)

r_{PHA} – Volumetric productivity (g_{PHA}/L.h)

r_s – Global substrate uptake rate (g_s/L.h)

rpm – Rotation per minute

T_g – Glass transition temperature (ºC)

T_m – Melting temperature (ºC)

vvm – Gas volume per liquid volume per time (minutes)

X - Active biomass concentration (g/L)

$Y_{PHA/s}$ – Storage yield (g_{PHA}/g_s)

$Y_{x/s}$ – Growth yield (g_x/g_s)

μ_{max} – Maximum specific growth rate (h^{-1})

ΔH – Melting enthalpy (J/g)

ΔH_f – Melting enthalpy of 100% crystalline P(3HB) (J/g)

χ_c – Crystallinity (%)

Abbreviations

AP – Peak areas ranging from C14:0 to C18:3

API – Peak area of methylheptadecanoate

BOD – Biochemical Oxygen Demand (ppm)

COD – Chemical Oxygen Demand (ppm)

C_{titr} – Concentration of titrant (g/L)

DO – Dissolved oxygen (%)

FDA – U.S. Food and Drug Administration

FFAs – Free fatty acids

FW – Fat wastes

FW_m – Mass of fat wastes after lyophilization (g)

$FW_m(H_2O)$ – Mass of fat wastes (g)

HV – 3-Hydroxybutyrate

HD – 3-hydroxydecanoate

HO – 3-hydroxyoctanoate

HHD – 3-Hydroxydodecanoate

HHp – *3*-hydroxyheptanoate

HHx – 3-hydroxyhexanoate

HTD – 3-hydroxytetradecanoate

HV – Hydroxyvalerate

GC – Gas chromatography

LB medium – Luria-Bertani medium

M – Molar weight of oleic acid

m_1 – Mass of tube with dried biomass (g)

m_2 – Mass of the empty tube (g)

mcl-PHAs – Medium-chain-length PHAs

m_{PHA} – Mass of PHA (g)

m_{cells} – Mass of liophylized biomass (g)

m_{film} – Mass of the polymer film obtained after extraction (g)

m_{PHAmax} – Maximum amount of PHA produced (g)

m_{ms1} – Mass of tube with dried margarine waste (g)

m_{ms2} – Mass of the empty tube (g)

m_s – Mass of residual margarine waste (g)

m_{si} – Mass of the initial margarine waste (g)

m_{sf} – Mass of the final margarine waste (g)

m_X – Mass of liophylized biomass (g)

m_{Xmax} – Maximum amount active biomass (g)

n.a. – Data not available

P(3HB) – Poly(3-hydroxybutyrate)

P(3H-co-HV) or PHBV – Poly (3-hydroxybutyrate-co-3-hydroxybutyrate)

P(3H-co-4HB) – Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)

PHA – Polyhydroxyalkanoate

PHBH – Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

P_m – Mass of dried pellet (g)

scl-PHAs – Short-chain-length PHAs

$SFWB_m$ – Mass of syringe with fat wastes (g)

SE_m – Mass of the empty syringe (g)

S_m – Mass of dried supernatant (g)

V_{titr} – Total volume of titrant (mL)

V – Volume

wt – Weight

W_{smp} – Fat waste weigh (g)

1 Motivation

Plastic materials are present in everyday life in many forms, like utensils, packaging, furniture, machinery housings and accessories, so they are used in almost all industries (Khanna & Srivastava, 2005). Over the past few years, the use of plastics has grown extensively: about 200 million tons of plastic materials, predominately petroleum derived, are consumed yearly worldwide (Gironi & Piemonte, 2011). The reduction of crude oil resources causes an increase of conventional plastics prices. Additionally, those plastics are discarded in the environment, where they accumulate due to their non-biodegradability. On the other hand, their disposal by incineration contributes to greenhouse gas emission and has high costs. Recycling processes are a valuable alternative, but not the ideal solution, due to the difficulty in sorting the different plastic materials and the impact on the properties of the recycled materials (Castilho et al., 2009; López-Cuellar et al., 2011).

Thereby, there is a need for sustainable raw materials to replace fossil resources (Du et al., 2012). In this context, polyhydroxyalkanoates (PHAs) are among the top group of biopolymers that have been intensively investigated and are already being commercialized. They are completely biodegradable under aerobic and anaerobic conditions and possess properties similar to various synthetic thermoplastics. Moreover, they derived from sustainable biomaterials and are synthesized by numerous microorganisms (Castilho et al., 2009; Khanna & Srivastava, 2005). However, PHAs are still not competitive enough to replace the conventional plastics due to their high production costs, which are mainly related to the high price of the carbon source. The use of wastes generated by agriculture and industrial processes for PHA production arises as a good alternative for reduction the overall production costs of the polymer and is advantageous for waste management.

For this purpose, the objective of this work was the production of PHA using fat-containing wastes generated by the food industry as the carbon source. The bioprocess developed allows the economic valorisation of that residue, which needs to be treated before disposal, with efficient production of value-added PHAs. Firstly, the fat materials were characterized and several bacteria were screened for their ability to produce PHA from those wastes. An optimization of bioprocess using the selected bacterial strain was carried out using different strategies to improve polymer yield and productivity. The resulting PHA polymers were characterized.

2 Introduction

2.1 – Fat wastes

Vegetable oils and fats are the most important source of fat in the human diet (Strayer et al., 2006). Derived from an array of vegetable sources (soybean, canola, cottonseed, corn, etc.), oils are extracted and processed for a variety of food uses like frying the oil. They are mainly composed of triglycerides (95%), which result from the combination of one unit of glycerol and three units of fatty acids, and small amounts of mono and diglycerides (Reda & Carneiro, 2007). Depending on the amount of saturated or unsaturated fatty acids, they may be solid, semi-solid or clear liquid materials at ambient temperature. Oils contain one to four double bounds in the carbon chain and because of the presence of unsaturated fatty acids they are in liquid form at ambient temperature. Due to high content of saturated fatty acids, fats have high melting points and are usually solids at room temperature. Oils and fats are insoluble in water but soluble in most organic solvents (e.g. hexane, acetone or ethanol) and they have densities lower than water. Besides, mono-, di- and triglycerides, oils and fats contain minor components, such as phosphatides, cerebrosides, sterols, terpenes, fatty alcohols, free fatty acids and vitamins (Strayer et al., 2006).

FIMA SA – Unilever, Portugal, uses a Oils and Fats Refining Process for the production of margarine, creams, emulsified chocolate and culinary broths. Their global annual production is about 47,000 ton (37,297 ton of margarine, creams and emulsified chocolate, and 9,433 ton of culinary broths).

For margarine production, the raw materials required are crude, refined or semi-refined oils that are treated in the refinery after coming to the manufactory. The main vegetable oils used in the process are sunflower, corn, soybean, coconut and palm oils, palm kernel, coconut oil and palm oil derivatives (stearin and olein oil). The main fatty acid composition of those vegetable oils is given in Table 2.1.

Table 2.1 – Fatty acid composition (wt%) of several vegetable oils (Adapted from Zambiazi et al., 2007).

Fatty acid	Carbon number	Sunflower	Corn	Soybean	Palm	Coconut	Palm Kernel
Caprilic	8:0	-	-	-	-	6.38	3.43
Capric	10:0	-	-	-	-	5.56	3.23
Lauric	12:0	-	-	-	-	45.46	46.14
Myristic	14:0	0.06	-	0.06	1.12	18.82	16.17
Palmitic	16:0	5.70	10.34	9.90	42.70	10.08	8.65
Palmitoleic	16:1	-	-	0.08	-	-	-
Stearic	18:0	4.79	2.04	3.94	4.55	4.31	2.27
Oleic	18:1	15.26	25.54	21.35	39.37	7.45	16.46
Linoleic	18:2	71.17	59.27	56.02	10.62	1.80	2.76
Linolenic	18:3	0.45	1.07	7.15	0.21	-	-
Arachidic	20:0	0.30	0.44	0.41	0.39	0.08	0.15
Gadoleic	20:1	0.22	0.37	0.22	0.17	0.06	0.17
Eicosadienoic	20:2	0.09	0.09	-	-	-	-
Behenic	22:0	1.16	0.31	0.48	0.58	-	-
Lignoceric	24:0	0.31	0.26	0.21	0.06	-	0.30

Following a quality control, crude oils are subjected to a series of sequential steps from refinery process (Figure 2.1):

1) neutralization with sodium hydroxide (caustic refining) to remove free fatty acids, phosphatides and other materials (e.g. protein meal, glycerol, carbohydrates, resins and metals);

2) bleaching to reduce colour pigments, as well as removal of oxidation products and residual neutralizing soap. This process employs bleaching earth as an adsorbent;

3) deodorization (vacuum steam distillation process) for removal of trace volatile components (e.g. aldehydes and ketones) that give rise to undesirable flavours, colours and odours (Karasulu et al., 2011).

4) margarine production, where the fat phase is prepared by adding vitamins, natural colourings, flavouring in the pre-treated oil to improve the quality and enhance flavour. Also emulsifiers are added to stabilize the emulsion.

When required, the pre-treated oils may follow parallel paths:

5) hydrogenation is used to provide the desired stability and functionality to edible oil products. The process consists of addition of molecular hydrogen to unsaturation points in the fatty acids, eliminating double bonds and making them into partially or completely saturated fats. There is generally a gradual increase in the melting point of the fat or oil, thus increasing their oxidative and thermal stability (Strayer et al., 2006);

6) interesterification, which causes a redistribution of the fatty acids across the glycerol backbone of the triglyceride. This process is carried out by adding a catalyst (sodium methylate) that is neutralized and reused. The rearranged product is washed, bleached, and deodorized to give rise to final oil product with different characteristics than the original oil blends.

Neutralization and interesterification processes are done at temperatures within the range 85-105ºC.

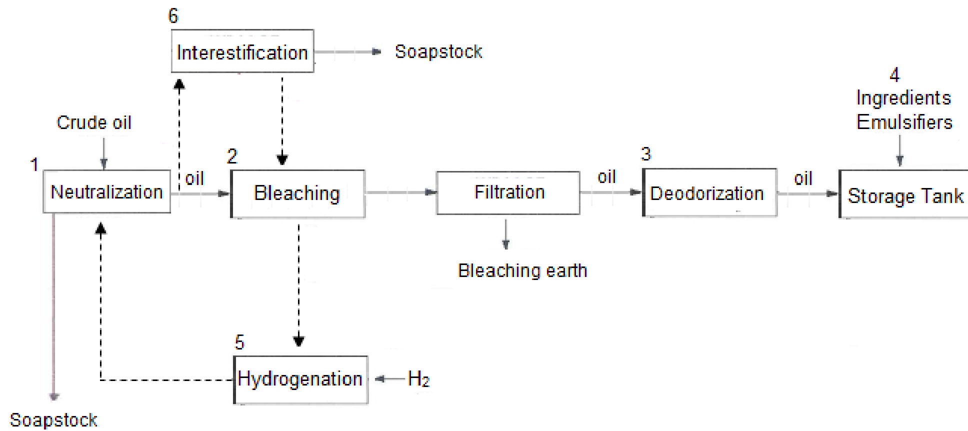

Figure 2.1 – Flowsheet of the refinery process used by FIMA.

FIMA has a specific installation called Cleaning in Place (CIP) to perform the cleaning of production lines, which contains all the oils not used. Figure 2.2 shows the flowsheet of soapstock treatment and the wastewater treatment plant. Soapstock and other impurities produced during the neutralization and interesterification processes are removed from the oil stream by fatty acids regeneration. Sulphuric acid (96%) is added to the soapstock and impurities solution, at 90ºC with stirring, to neutralize the reaction mixture that is separated by settling (1 and 2). The wastewaters generated by soap splitting and CIP and margarine production are treated at a wastewater treatment plant. The first treatment (4 and 5) is performed by a gravity separator and consists of removing free fatty acids from the wastewater and collecting them in separated tanks. Fats from CIP and margarine production (margarine waste) and soap spliting (refinery waste) are stored in tanks and sold to oil-recycling companies (3). In some cases, the wastewater from margarine and refinery waste can be mixed when they overflow (6). The sewage water passes through the homogenizer tank to collect the wastewater (7) and, then, the large size pollutants are removed by the addition of a flocculating agent in dissolved air flotation process (DAF) (8). The effluents from other process are also subject to DAF process (9). The resultant effluent flows to a storage tank, resulting sludge. Therefore, three types of fat material are generated at FIMA manufacture: fats from margarine and refinery wastes and sludge. In 2012, 279 ton of fats of margarine and refinery waste were produced. The annual production of sludge is about 1000 ton and their treatment has costs.

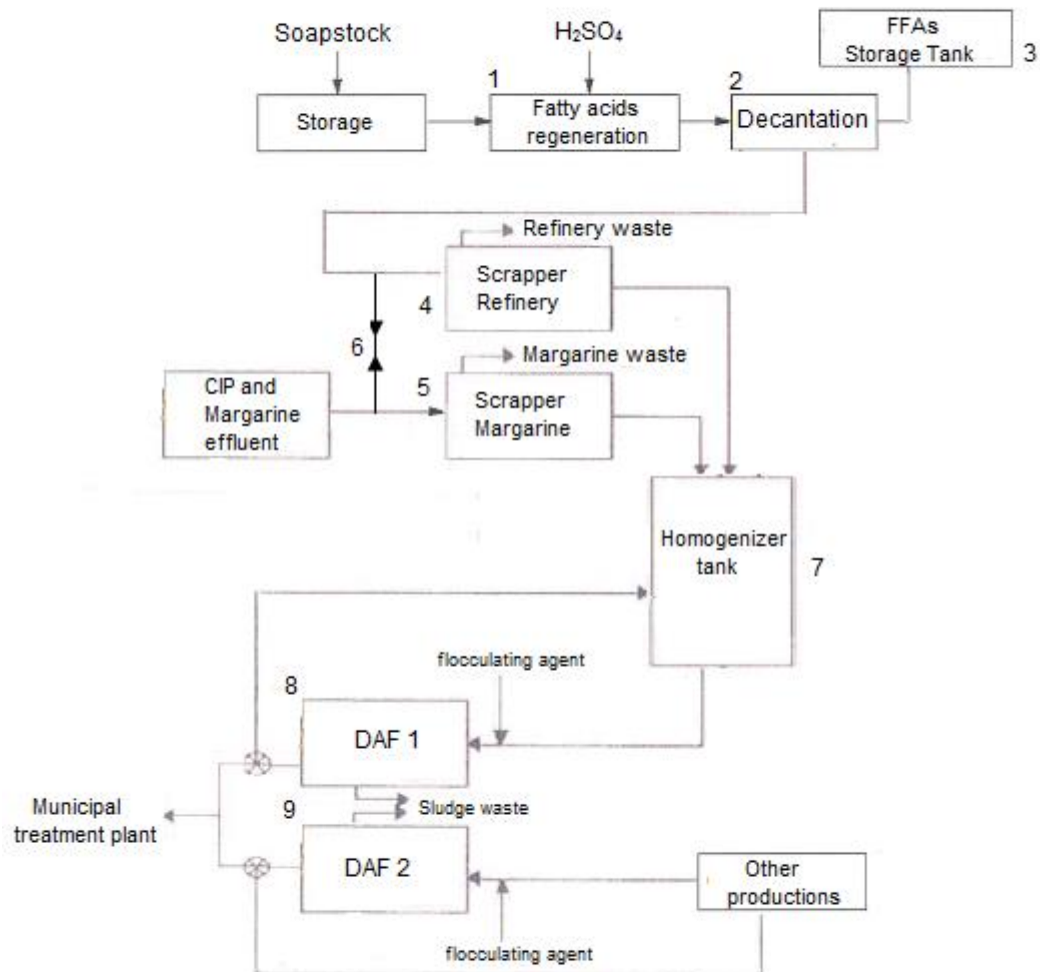

Figure 2.2 – Flowsheet of soapstock treatment and wastewater treatment plant at FIMA.

The raw wastes and wastewaters generated from the food industry are the major source of water and land pollution, because compounds with high lipid components are difficult to treat (Cammarota & Freire, 2006). An increase in the biological oxygen demand (BOD) and chemical oxygen demand (COD) occurs when improper disposal of these wastes occurs. This situation leads to the deoxygenation of surface waters, infiltration into soil sediments and aquifer contamination (Akaraonye et al., 2010). Treatment of these wastes is associated with high costs of management, so their recycling is of great importance for the manufacturers (Taniguchi et al., 2003). The valorization of waste oils and by-products not only avoids environmental pollution and decreases the disposal costs, but also allows for the production of value-added products (Castilho et al., 2009).

2.2 – Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are polymers of hydroxyalkanoic acids that are synthesized by many microorganisms as intracellular carbon and energy reserve materials or reducing-power storage materials. Numerous prokaryotic microorganisms accumulate PHA

under conditions of nutrient limitation, such as nitrogen or phosphorus, and in the presence of excess carbon source (Lee, 1996a). Hence, PHAs are accumulated in the cytoplasm as densely packed water insoluble granules. When the limiting nutrient is provided to the cell, these energy storage compounds are degraded and used (Höfer et al., 2011). The PHA granules consist of 97.7% PHA, 1.8% protein and 0.5% lipids (Koller et al., 2010).

PHAs have received extensive attention mainly due to two interesting properties: biodegradability and biocompatibility (Akaraonye et al., 2010). These polymers have the capacity to be degraded by microorganisms into water-soluble oligomers and monomers (3-hydroxybutyric acid, a known constituent of blood plasma) that are used as carbon source by microorganisms in the natural environment (Ojumo et al., 2004). Also, it is important to refer that PHAs are non-toxic in nature, presenting the advantage of being biocompatible, features that render them suitable for use in many medical applications. Furthermore, these polymers possess physical-chemical characteristics similar to traditional plastics, but have the considerable advantage of being biocompatible and biodegradable (Lee, 1996a).

The general structure of PHAs (Figure 2.3) can be manipulated by genetic or physiological strategies. The pendant group R varies from methyl (C1) to tridecyl (C13), that influence the physical properties of the polymers, such as melting temperature, glass transition temperature and crystallinity (Lee, 1996b). The biodegradability and biocompatibility of PHAs are influenced by the sterospecific incorporation of R(-) monomer and these monomers can have saturated, unsaturated, straight or branched side-chain (Koller et al., 2010). PHAs have a high degree of polymerization, which is related with molecular weight, are highly crystalline, optically active, isotactic and piezoelectric (Reddy et al., 2003). Depending on the microorganism and the growth conditions, the molecular weight of these compounds varies in the range of 2×10^5 to 3×10^6 (Khanna & Srivastava, 2005).

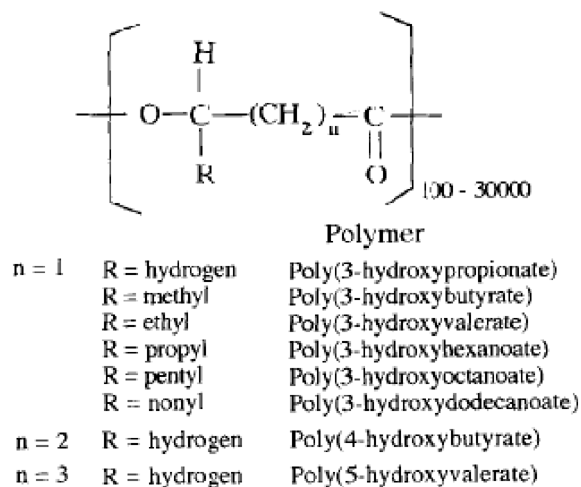

Figure 2.3 – General structure of polyhydroxyalkanoates (Lee, 1996a).

The lengths of the side chain and functional group have a great importance on the physical properties. Based on the length of the side chain, PHAs can be classified as short-chain-length (scl) or medium-chain-length polymers (mcl). Scl-PHAs include polyesters

containing 3–5 carbon atoms and they are crystalline, britle and stiff polymers, with high melting point and low glass transition temperature, e.g. P(3HB), poly(3-hydroxyvalerate) [P(3HV)] or the copolymer P(3HB-co-3HV) (Lee, 1996a). In contrast, mcl-PHAs include those polymers formed by monomers equal to or longer than C6, exhibiting low crystallinity and tensile strength but high elongation to break, lower melting points and glass transition temperatures (Akaraonye et al., 2010). Table 2.2 presents physical, mechanical and thermal properties of commercialized PHAs comparing with polypropylene.

Table 2.2 – Comparison of the physical properties of scl-PHAs and mcl-PHAs with polypropylene (Adapted from Koller et al., 2010).

Properties	P(3HB)	P(3HB-co-3HV)	mcl-PHAs	Polypropylene
Crystallinity (%)	40–80	55 – 70	20–40	70
Melting point (ºC)	171–182	75–172	30–80	176
Density (g cm^{-3})	1.25	1.20	1.05	0.91
Tensile strength (MPa)	40	25–30	20	34
Glass transition temperature (ºC)	5–10	-13–8	-40–150	-10
Elongation at break (%)	6	8–1200	300–450	400
UV light resistance	Good	Good	Good	Poor
Solvent resistance	Poor	Poor	Poor	Good
Biodegradability	Good	Good	Good	None

Today, approximately 150 hydroxyalkanoic acids have been reported as monomers of polyhydroxyalkanoates (Steinbuchel, 2001). P(3HB) was the first PHA to be discovered and is the most widely studied and best characterized PHA. It is a homopolymer of 3-hydroxybutyrate, which has mechanical properties similar to polypropylene, like others scl-PHAs (Lee, 1996a). The molecular weight of P(3HB) produced from wild-type bacteria (e.g. *Cupriavidus necator*) is usually in an interval of 10^4–10^6 with a polydispersity index around (Khanna & Srivastava, 2005; López-Cuellar et al., 2011). It has a glass transition temperature (Tg) in range of 5–10ºC, whereas its melting point occurs at ~180ºC, similar to that of polypropylene (176ºC) (Table 2.2). But P(3HB) has still not been used in many industrial applications due to its low thermal stability and excessive brittleness upon storage (elongation at break of 6%) (Koller et al., 2010; Lee, 1996a).

The polymer properties can be improved with the incorporation of monomer units such as 3-hydroxyvalerate or 4-hydroxybutyrate to form copolymers, like P(3HB-co-3HV) which have lower crystallinity and decreased stiffness, being more flexible and tougher than P(3HB) (Lee, 1996a).

On the other hand, mcl-PHAs have gained much interest in research on biopolymers, because of their ability of chemical modification which have shown promise for medical applications (Sun et al., 2007).

2.3 – Applications

Due to their biocompatibility and biodegradability, PHAs present a great potential to be used in several fields, such as packaging, agricultural, fisheries and medical applications. PHAs have been used as packaging materials and shampoo bottles by Wella AG, Germany. Other products have also been developed, including packaging films (e.g. bags, paper coatings) and other products like razors, utensils, diapers, feminine hygiene products, cosmetic containers and cups (Chen, 2009).

There is a high interest in the use of PHA in medicine. Their biocompatibility with a wide range of tissues was demonstrated in various tests using animal models (Valappil et. al, 2006). In 2007, the U.S. Food and Drug Administration (FDA) approved the use of P(4HB) for surgical sutures in clinical applications, leading to enhancement of the use of PHA in medicine. Fixation and orthopaedic applications include sutures, regeneration devices, fixation rods, bone plates and bone fracture fixation, ligament and tendon grafts, bone graft substitutes and the fabrication biodegradable heart valve scaffolds (Valappil et. al, 2006).

PHAs can be used in the synthesis of chiral compounds, such as enantiometrically pure chemicals, and for the production of paints (Reddy et al., 2003). Besides that, it can be applied in foils, films and performance additives, such as surface treatment of paper, replacing the conventional petrochemical products (Akaraonye et al., 2010; Bourbonnais & Marchessault, 2010). They can also be used as biodegradable carriers of drugs, hormones, insecticides and herbicides (Reddy et al., 2003).

In the food industry, there is much interest in PHAs as raw material in many applications, such as edible packaging material, coating agent, flavour delivery agent or dairy cream substitute in the food industry (Chakraborty et al., 2012).

2.4 – PHA-producing bacterial strains

More than 300 species of bacteria from Halobactericeae and Archaea families synthesize PHAs. However only a few bacteria can be cultivated to high cell densities with a high PHA content in a relatively short period of time (Lee & Choi, 1998). Some factors should be considered in the choice the microorganism, including the cell's ability to utilise an inexpensive carbon source, growth rate, polymer synthesis rate, and the maximum extent of polymer accumulation (Khanna & Srivastava, 2005). *Cupriavidus necator, Rhodopseudomonas palustris, Methylobacterium organophilum* and *Pseudomonas* sp. are examples of bacteria that accumulate PHAs under unfavorable growth conditions (Akaraonye et al., 2010). But, some

bacteria have the ability to accumulate PHA under non-limiting conditions, e.g. *Alcaligenes latus* and recombinant *Escherichia coli* (Akaraonye et al., 2010;Lee, 1996a).

Microorganisms are capable of producing PHA from various carbon sources, ranging from inexpensive, complex waste effluents to plant oils and their fatty acids, alkanes and simple carbohydrates (Sudesh et al., 2000). Sugars are the most commonly used substrates, but the cost of this type of carbon source contributes largely to the high price of PHAs (Choi & Lee, 1999). For a sustainable biopolymers production, Kim (2000) examined inexpensive renewable carbon sources as PHA carbon feedstock and concluded that their use can reduce the overall production costs in about 40–50%. Fatty acids and vegetable oils are advantageous compared with other substrates for large-scale PHA production. The theoretical yield coefficient of PHA from vegetable oils (e.g. 1.38 g PHA per 1 g of linoleic acid) was higher than the yield from glucose (0.48 g PHA per 1 g of glucose), because they contain much higher carbon content per weight than sugars (Akiyama et al., 2003). Besides, wastes and by-products rich in oils are better alternatives when compared with food grade oils. Production of PHA from these substrates may cause an imbalance in food supply and depletion of food sources, which is a social concern. For this goal, fats/oils and their recycled products are potentially useful feedstocks for fermentation processes (Sudesh et al., 2011). A summary of PHA produced from various vegetable oil and animal fats using wild-type bacteria in Table 2.3.

Table 2.3 – Production of PHA by various bacteria from different vegetable oils and fats, n.a.: data not available.

Strain	Operation Mode	Carbon Source	Initial Carbon Source (g/L)	CDW (g/L)	PHA (g/L)	PHA content (%, wt/wt)	Productivity (g_{PHA}/L.h)	PHA Composition	References
C. necator DSM 428	Fed-Batch	Soybean Oil	20	126	95.8	76	1.00	P(3HB)	Kahar et al. (2004)
C. necator DSM 530	Batch	Soybean Oil	20	15	13.0	83	0.15	P(3HB)	Park & Kim (2011)
C. necator DSM 428	Shake flask	Vegetable oils	10	3.6–4.3	2.9–3.4	79–82	0.04–0.05	P(3HB)	Fukui et al. (1998)
C. necator DSM 428	Fed-Batch	Jatropha Oil	20	65.2	49.6	76	1.03	P(3HB)	Ng et al. (2010)
C. necator DSM 428	Shake flask	Waste frying rapeseed oil	20	3.7	1.2	32	0.02	P(3HB)	Verlinden et al. (2011)
C. necator DSM 428	Three-stages	Canola oil	75	19.9	18.3	92	0.4	P(3HB), mcl-PHA	López-Cuellar et al. (2011)
C. necator DSM 428	Batch	Emulsified plant oil	17 (0.5% GA)	10	7.9	79	0.11	P(3HB)	Budde et al. (2011a)
C. necator DSM 428	Shake Flask	Waste plant oils and tallow	10	4.8–7.3	3.1–5.8	57–80	0.04–0.08	P(3HB/3HV)	Taniguchi et al. (2003)
C. necator DSM 428	Shake Flask	Waste cooking oil	20	25.4	18.0	71	0.25	P(3HB)	Kamilah et al. (2013)
C. necator DSM 428	Batch	Methylated oils	2.5	2–40	0.9–1.0	3–45	0.01– 0.03	P(3HB/3HV)	Kocer et al. (2003)
P. resinovorans NRRL B-2649	Shake Flask	Vegetable oils Animal fats	5	2.9–3.8	1.2–1.9	40–51	0.02–0.04	mcl-PHA	Ashby et al. (1998)
P. oleovorans NRRL B-14682	Shake Flask	CSBP[a]	50	1.3	0.4	27	0.006	P(3HB)	Ashby et al. (2004)
P. oleovorans NRRL B-14683	Shake Flask	Tallow free fatty acids	2.4–2.7	1.6	0.3	18	0.006	mcl-PHA	Cromwick et al. (1996)

Table 2.3 (Cont.).

P. resinovorans NRRL B-2649	Shake Flask	Tallow free fatty acids Tallow	2.4–2.7	0.9–1.3	0.1–0.2	14.6–15.1	0.002 – 0.004	mcl-PHA	Cromwick et al. (1996)
P. citronellolis NRRL B-2504	Shake Flask	Tallow free fatty acids	2.4–2.7	1.7	0.05	3	0.001	mcl-PHA	Cromwick et al. (1996)
P. citronellolis NRRL B-2504	Fed-batch	SFAE[b]	5–7.5	30.6–42	2.8–2.9	20–27	0.067–0.1	mcl-PHA	Muhr et al. (2013)
P. stutzeri NRRL B-1317	Shake Flask	Soybean oil	10	2.7	1.7	63	0.04	mcl-PHA	He et al (1998)
P. oleovorans NRRL B-778	Batch	linoleic acid, methylated oils	2.5	0.6–2.3	0.3–1.9	7–29	0.01–0.08	mcl-PHA	Kocer et al. (2003)
C. testosteroni NRRL B-2611	Shake Flask	Vegetable oils	20	n.a.	n.a.	79–88	n.a.	mcl-PHA	Thakor et al. (2005)

[a] Co-product stream from soy-based biodiesel production containing glycerol, fatty acid soaps, and residual fatty acid methyl esters.
[b] Saturated biodiesel fractions originating from animal waste lipids.

Cupriavidus necator (formerly known as *Ralstonia eutropha*) is an aerobic, chemo-organotrophic β-proteobacterium, known as the model organism for PHA production, because of its ability for accumulation of high amounts of polymer (up to 90%), using simple carbon sources, such as glucose, lactic acid and acetic acid (Riedel et al., 2012). This bacterium was also reported to be able to accumulate P(3HB) up to approximately 80% (wt/wt) of the cell dry weight from plant oils, as shows Table 2.3 (Fukui & Doi, 1998; Kahar et al., 2004). Since plant oils were difficult to be efficiently used due to the heterogeneity of the two phase aqueous medium, the substrate was emulsified in the growth medium using gum arabic as the emulsifying agent (Budde et al., 2011a). Also, waste oil can become a good alternative compared with pure oil or glucose as substrates for cultivation of PHA-producing bacteria. Verlinden et al. (2011) reported the production of 1.2 g/L P(3HB) from waste frying oil by *C. necator* (Table 2.3), which was similliar to the production obtained from glucose by the same strain. Wild-type *C. necator* is widely used in industrial processes to produce PHA, therefore there have been many studies to produce high PHA yield using mutant strains (Bhubalan et al., 2008; Budde et al., 2011b).

Bacteria of the genus *Pseudomonas* are known for their ability to synthesize medium-chain-length PHA (Lageveen et al., 1988). This genus belonging to rRNA homology group I, which accumulates mcl-PHA when cultivated on various aliphatic alkanes or aliphatic fatty acids (Huisman et al., 1989). Among the PHA-producing *Pseudomonas* species, *P. oleovorans* NRRL B-14683, *P. resinovorans* NRRL B-2649, *P. putida* KT2442, and *P. citronellolis* were reported to produce PHA from tallow, a cheap fat (Cromwick et al., 1996). Table 2.3 shows that several *Pseudomonas* strains are able to grow and accumulate P(3HB) and mcl-PHAs using triglyceride substrates, fatty acids and co-products stream from soy-based biodiesel production (CSBP) (Ashby & Foglia., 1998; Ashby et al., 2004; He et al., 1998).

Comamonas testosteroni is also a promising candidate for PHAs production on different carbon sources. It was studied by Thakor et al. (2005) for its ability to synthesize mcl-PHAs during cultivation on vegetable oils, accumulating up to 78.5–87.5% (wt/wt) of the cellular dry material (Table 2.3).

PHA synthesis is determined not only by the producing microorganism and the carbon sources used, but also by the metabolic routes involved (Lageveen et al., 1988). Many long-chain fatty acids can be used by microorganisms, in the presence of extracellular lipase that induces their enzymatic hydrolysis (Kahar et al., 2004). After the lipase action, the fatty acids are transferred through the cell membrane and metabolized via β-oxidation pathway to produce PHA monomers. Fats can be subjected to a pretreatment procedure, like saponification, so that fatty acids can more easily be incorporated by microorganisms, especially those that are not able to synthesize lipases (Kocer et al., 2003; Tan et al., 1997). There are three metabolic pathways (Figure 2.4) involved in the generation of PHA monomers. Control of these pathways is ensured by many genes that encode different enzymes (Sudesh et al., 2000). In *C. necator*, the synthesis of P(3HB) is a simple biosynthetic pathway that involves three enzymes and their encoding genes. In the first step, two acetyl-CoA molecules are condensed to form acetoacetyl-

CoA, a reaction catalyzed by β-ketothiolase, which is encoded by phaA gene. In the next step, acetoacetyl-CoA is reduced to (R)-3-hydroxybutyryl-CoA by the enzyme acetoacetyl-CoA reductase that is encoded by phaB gene and is NADPH-dependent. The last reaction, is the polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase (encoded by the phaC gene) (Lee, 1996a; Sudesh et al., 2000).

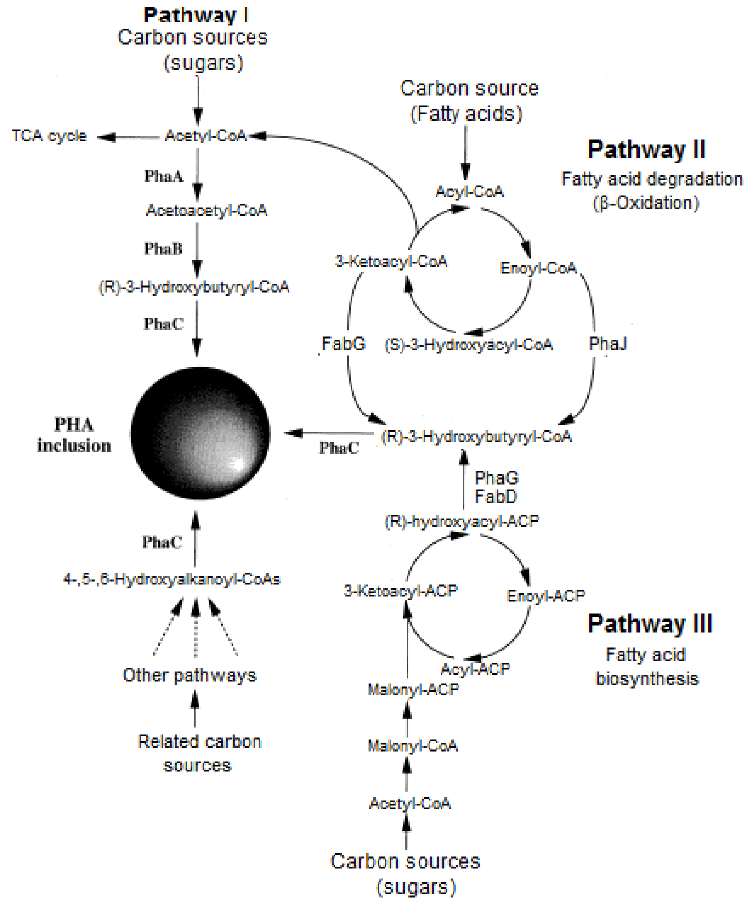

Figure 2.4 – Metabolic pathways involved in synthesis of PHA. PhaA, β -ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaG, 3-hydroxyacyl-ACP-CoA transferase; PhaJ, (R)-enoyl-CoA hydratase; FabD, malonyl-CoA-ACP transacylase; FabG, 3-ketoacyl-CoA reductase (Sudesh et al., 2000).

Another type of PHA biosynthetic pathway is exhibited by *Pseudomonas* species that derives the 3-hydroxyacyl-CoA to form mcl-PHAs, from the intermediates of fatty acid β-oxidation pathway, enoyl-CoA, 3-ketoacyl-CoA, and/or S-3-hydroxyalcyl-CoA. *Pseudomonas* species, except *P. oleovorans,* can also synthesize mcl-PHAs from unrelated carbon sources such as carbohydrates, where the 3-hydroxyacyl monomers are derived from the de novo fatty acid biosynthesis pathway (Sudesh et al., 2000).

2.5 – Production of PHAs in Bioreactors

The production of PHA by microorganisms using different types of cultivation modes has been studied for PHA production optimization. Batch and fed-batch fermentations are widely used in the industrial PHA production (Chee et al., 2010).

There are many factors that influence the bioprocess, namely the physiological requirements of the microorganism, such as the temperature range, stirrer speed, dissolved oxygen concentration, pH and substrate composition and concentration (Tajalli & Roy, 2010). Batch cultures have been employed in the investigation and optimization of scl-PHAs and mcl-PHAs (Akaraonye et al., 2010). But this technique is not ideal to PHA accumulation, because after the onset of nitrogen limitation the cell viability decrease and the cells are not fully active and deteriorate partially (Akaraonye et al., 2010). Fed-batch fermentation is a classical strategy in PHA production and one of the best methods used to ensure large amounts of polymer accumulation within a shorter time span. For the fed-batch cultures, a two stages cultivation method is most often employed. The main aim is to achieve a high cell density culture in the first stage and, then, to increase the concentration of PHAs in a second stage by a nutrient limited cultivation. In this context, this type of cultivation is suitable for bacteria that require limitation of essential nutrients for the efficient synthesis of PHA (Akaraonye et al., 2010; Khanna & Srivastava, 2005).

The nutrient concentration needs to be controlled, because a premature limitation of nutrient limits the cell concentration and affects the final PHA concentration and consequently a low PHA productivity. Also, if nutrient limitation is delay too long, cells are not able to accumulate, resulting in PHA content and a low PHA productivity (Lee, 1996b). A fed-batch strategy avoids problems of inhibition by substrate (López-Cuellar et al., 2011). Besides, in fed-batch fermentation the optimal strategy is to feed the growth limiting substrates at the same rate as the rate of substrate utilization by organism. This avoids by-products production by limiting its quantity to the amount required for production of the product of interest (Akaraonye et al., 2010).

2.6 – Downstream processing

Due to its intracellular nature, PHA recovery process from the biomass is a rather costly process (Chee et al., 2010). Following biosynthesis process, cells containing PHAs are separated from the cultivation broth by conventional procedures, such as centrifugation, and then the harvested cells are lysed for PHAs recovery. Some factors have been considered when choosing the adequate method for PHAs recovery, namely the production strain, the required product purity, the availability of isolation agents and the acceptable impact on the molecular mass (Koller et al., 2010). In Table 2.4, different strategies used for PHA recovery from the biomass are summarized.

Most commonly used methods involve extraction using solvents, like chloroform, dichloromethane or 1,2-dichloroethane. Typically, the biomass is contacted with the organic solvent, extracting PHA by its dissolution in the solvent. After removing cell debris by filtration, the polyester is precipitated on a cold PHA antisolvent (e.g. ethanol and methanol) (Ramsay et al., 1994). The use of solvents is very simple and effective and can achieve highly pure PHA with high average molecular weight. Compared with other recovery methods, solvent extraction causes negligible degradation to the polymers and is able to remove bacterial endotoxin, making PHA suitable for medical applications. On the other hand, the solvent destroys the natural morphology of PHA granules, which is advantageous for some applications, like the production of strong fibbers, but disadvantageous for applications wherein amorphous polymer is necessary (e.g. paper coating) (Bourbonnais & Marchessault, 2010; Jacquel et al., 2008). Nevertheless, large amounts of toxic and volatile solvent are required, so that the extracted polymer solution is not too viscous and the removal of cell debris is more effective. This large quantity of solvent is not only an hazardous to human health and the environment, but also increases the total production costs of PHAs (Choi & Lee, 1997).

In order to overcome these problems, other methods to recovery PHA has been studied, based on digestion of non-PHA cellular materials by using chemical (e.g. sodium hypochlorite) or enzymatic digesters (e.g. protease or lysozyme) (Table 2.4). A range of surfactants have been evaluated for their ability of digestion of cellular materials and, among them, SDS showed good performance (Chen, 2009; Ramsay et al., 1990). Mechanical cell disruption, dissolved air flotation or extraction using supercritical CO_2 have also been recently reviewed as potentially good alternatives for PHA recovery (Hejazi et al., 2003; Tamer & Moo-Young, 1998).

Table 2.4 – Some of polyhydroxyalkanoates isolation methods, n.a.: data not available.

Method	Strain	Results	Reference
Solvent extraction **Chloroform**	*Cupriavidus necator* DSM 545	Purity: 95%; Yield: 96%	Fiorese et al. (2009)
1,2-Propylene carbonate	*C. necator* DSM 545	Purity: 84%; Yield: 95%	Fiorese et al. (2009)
Methylene chloride	*C. necator* DSM 428	Purity: 98%	Zinn et al. (2003)
Chemical digestion **Surfactants** **Palmitoyl carnitine**	*C. necator*	Release rate >85%	Lee et al. (1993)
Sodium hypochlorite	*C. necator* DSM 545	Purity: 98%	Berger et al. (1989)
Surfactant-sodium hypochlorite **Triton X-100-sodium hypochlorite**	*C. necator* DSM 545	Purity: 98%	Ramsay et al. (1990)
Surfactant-Chelate **Betaine-EDTA disodium salt**	*C. necator* DSM 545	Purity: >96%; Yield: 90%	Chen et al. (1999)
Selective dissolution by protons **Sulfuric acid**	*C. necator*	Purity: >97%; Yield: >95%	Yu & Chen (2006)
Enzymatic digestion **Bromelain; pancreatin**	*C. necator* DSM 545	Purity: 89%; Purity: 90%	Kapritchkoff et al. (2006)
Mechanical disruption **Bead mill** **High pressure homogenization**	*A. latus* *A. latus*	n.a.	Tamer & Moo-Young (1998)
Supercritical fluid **SC-CO_2**	*C. necator*	Yield: 89%	Hejazi et al. (2003)
Dissolved air flotation **Enzymatic hydrolysis, sonification, flotation**	*P. putida*	Purity: 86%	Hee et al. (2006)
Air classification	*C. necator*	Purity: 95%; Yield: 85%	Jacquel et al. (2008)

2.7 – Sustainability and environmental impact of PHAs

Regarding industrial PHA production, there are several companies producing PHAs on a large scale, mainly P(3HB) and P(3H-co-BV). Some PHA products in the markets are described in Table 2.5. But the industrial production is still small (50,000 ton per year) when

compared to the conventional plastics market, estimated at 150 million ton per year (Castilho et al., 2009). The major drawbacks in the production of PHA are the cost associated with substrates and the extraction of the polymer from the cells. Factors like PHA productivity, PHA content and PHA yield are all important factors to be optimized (Choi & Lee, 1999).

Table 2.5 – The current and potential large volume manufacturers of polyhydroxyalkanoates, n.a.: data not available (Adapted from Kosior et al., 2006).

Polymer	Trade names	Manufacturers	Capacity (tons)	Price (kg^{-1}) (in 2010)
P(3HB)	Biogreen®	Mitsubishi Gas Chemical Company Inc. (Japan)	10,000	€2.5–3.0
P(3HB)	Mirel™	Telles (US)	50,000	€1.5
P(3HB)	Biocycle®	P(3HB) Industrial Company (Brazil)	50	n/a
PHBV and P(3HB)	Biomer®	Biomer Inc. (Germany)	50	€3.0–5.0
PHBV, PHBV+ Ecoflex blend	Enmat®	Tianan Biologic, Ningbo (China)	10,000	€3.26
PHBH	Nodax™	P&G (US)	20,000–50,000	€2.5
PHBH	Nodax™	Lianyi Biotech (China)	2000	€3.7
PHBH	Kaneka PHBH	Kaneka Corporation (Japan)	1000	n.a.
P(3HB-co-4HB)	Green Bio	Tianjin Gree Bio-Science Co/DSM	10,000	n.a.
P(3HB-co-4HB)	Meredian	Meredian (US)	272,000	n.a.

The nature and availability of the substrate influence the cost of PHAs production, due to the expensive price of raw materials, especially the carbon source. About 40% of the total production cost is for raw material (Choi & Lee, 1999). Therefore, the production of a variety of PHAs by different bacteria and a final product that is economically competitive with traditional plastics with high volumetric productivity depend on the choice of media. The selection of the substrate should not focus only on the market prices but also on availability (Akaraonye et al., 2010).

Many studies of PHAs have showed a concern about their high production costs and environmental impact compared with petrochemical-based polymers synthesizes (Choi & Lee, 1999). Comparative studies of life cycle assessment of PHAs versus oil-derived plastics were done to evaluate the environmental impacts of PHA production (Gomez et al., 2012). These researches concluded that the dominant contributor is the energy requirements for sterilization, aeration, and agitation. Also, when carbon sources from agriculture feed-stocks are used, the energy to produce their substrates have to be considered (Gomez et al., 2012). However, using renewable resource, like oils and fats wastes, improves PHA manufacturing because energy and greenhouse-gas emissions are reduced (Hassan et al., 2012).

The optimization of the process is needed to reduce PHAs costs, because their price is still high (US $ 1.99/kg, in 2010) (Kosior et al., 2006) compared with conventional plastics, which is less than US $ 1/kg (Choi & Lee, 1997). For this purpose, the analysis of the entire process for the production and recovery of PHA should to be done to design the most efficient method of PHA production and to evaluate the approximate price of PHA produced on a commercial scale. Development of efficient metabolically engineered strains capable of utilizing cheap carbon sources, efficient cultivation strategies and improving the extraction and purification processes are necessary to make PHA attractive on the polymers market (Akaraonye et al., 2010; Jacquel et al., 2008).

3 Materials and Methods

3.1 – Fat wastes characterization

3.1.1 – pH measurement

The pH of fat wastes was determined at 50ºC, due to their solid consistence at ambient temperature, with pH electrode from Crison (basic 20 pH).

3.1.2 – Density

To determine the density, the three fat wastes (margarine, refinery and sludge) were melted at 70ºC and transferred (5 mL) into pre-weighed syringes. After cooling to room temperature the fat wastes were reweighed. The density (g/cm^3) was calculated by Equation 1. Duplicate analyses were performed for each sample.

$$Density\ (\mathrm{g/cm^3}) = \frac{\mathrm{SFWB_m - SE_m}}{\mathrm{V}} \quad (1)$$

$SFWB_m$ is the mass of syringe with fat wastes (g), SE_m is the mass of the empty syringe (g) and V is the volume of fat wastes (mL).

3.1.3 – Water content

To determine the water content, melted fat wastes (2 mL) were transferred into pre-weighed tubes and let to cool at room temperature. The fat wastes were frozen in liquid nitrogen and lyophilized (Telstar, Cryodos), for 2 days (temperature: -42 to -45ºC; pressure less than 0.35 mbar). The dried fat wastes were reweighed and the water content (%) was determined by Equation 2:

$$Water\ content\ (\%) = \frac{\mathrm{FW_m(H_2O) - FW_m}}{\mathrm{FW_m(H_2O)}} \quad (2)$$

where $FW_m(H_2O)$ is the mass of fat wastes (g) and FW_m is the mass of fat wastes after lyophilization (g). Triplicate analyses were performed for each fat waste.

3.1.4 – Solubility

The solubility of fat wastes in water and organic solvents (hexane (JMGS); ethanol, (Scharlau); acetone (JMGS)) was determined by mixing 4 mL of each sample in 40 mL of each solvent. The mixtures were stirred in the vortex (2500 rpm, 1 min, VWR) and centrifuged (16743 g, 15 min). The supernatant was transferred to pre-weighed plastic tubes and the solvent was evaporated in a fume hood until constant weight was obtained. The water mixtures were lyophilized for 2 days at a temperature range of -42 to -45ºC and pressure less than 0.35 mbar. The pellets were also dried in the fume hood until constant weight and weighed. The solubility of the fat wastes in each solvent was determined by Equation 3:

$$Solubility\ (\%\ extraction) = \frac{\mathrm{S_m}}{\mathrm{S_m+P_m}} \times 100\% \quad (3)$$

where S_m is the mass of dried supernatant (g) and P_m is the mass of dried pellet (g).

3.1.5 – Miscibility test

It was tested the miscibility of fat wastes in the mineral medium, which would be used in microbial cultivation (described in section 3.2.1). Each fat waste (2 mL) was mixed with 100 mL mineral medium, in 250 mL shake flasks and placed in an orbital shaker for three days at 200 rpm, at different temperatures (30ºC and 37ºC). The miscibility of the fat wastes in the mineral medium was evaluated by visual observation of the flasks. Then, the flasks were transferred to ambient temperature for a week to verify the mixtures' stability.

3.1.6 – Total sugar content

The fat wastes content in sugars was determined using the phenol-sulphuric acid method. 500 mg of each fat waste were added to 0.5 mL of 5 % phenol (Sigma) and 2.5 mL of concentrated sulphuric acid (Fisher). The mixtures were shaken for 10 min at 2500 rpm and after 30 min their optical density was measured at 490 nm (Hach spectrophotometer, Lange). A calibration curve was prepared using known concentrations of glucose (0–200 mg/L). The quantity of sugar was expressed as percentage of sugar in fat wastes. Duplicate analyses were performed for each sample.

3.1.7 – Inorganic content and composition

To determine the fat wastes composition in inorganic compounds, 1 mL of each fat waste was added to a pre-weighed porcelain crucible. The crucibles were placed in a preheated oven (Carbolite) at 550ºC, for 24h. Afterwards, the crucibles were placed in

desiccators to cool to room temperature and the ashes obtained were dissolved in 20 mL 2.3 M sulphuric acid solution. The solutions were analyzed by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES), in ICP Horiba Jobin-Y, France, Ultima, to quantify their content in aluminum, calcium, iron, magnesium, phosphorus, potassium and sodium.

3.1.8 – Elemental analysis

The carbon, hydrogen, nitrogen and sulphur content of each fat waste were analysed using the elemental Analyser Thermo Finnigan – CE Instruments (Italy), model Flash EA 1112 CHNS. Duplicate analyses were performed for each fat waste.

3.1.9 – Lipid profile analysis

For the analysis of glycerin, mono, di and triglycerides by gas chromatography, approximately 100 mg of each fat waste were accurately weighed in a 10 mL vial. Butanetriol (internal standard IS1) and tricaprin (internal standard IS2) solutions (Sigma and TCI, respectively) were prepared in pyridine (Carlo Erba) at concentrations of 1000 µg/mL and 8000 µg/mL, respectively, and used as internal standards. Four commercially available calibration solutions (Sigma), containing glycerin, monoolein, diolein, triolein, butanetriol (IS1) and tricaprin (IS2) were used to construct the calibration curves with concentrations in the range 5 – 1250 µg/mL (Table 7.1, section 7.1). Then, 0.8 µL of IS1, 100 µL of IS2 and 100 µL of the derivatization agent (N-Methyl-N-(trimethylsilyl) trifluoroacetamide, Acros organics) were added to the sample vial, which was hermetically sealed and shaken. After 15 min, 8 mL of n-heptane (Carlo Erba) was added and the final reaction mixture was injected (1 µL) into a Thermo Scientific TRACE GC Ultra equipped with a true cold On-column inlet and a flame ionization detector (FID) automated by a TriPlus Autosampler for liquids was used. A non-polar Thermo Scientific TRACE™ TR-BIODIESEL column (10 m, 0.32 mm, 0.1 µm) and a pre-column (1 m, 0.53 mm) was used, connected to the column by a leak-free high T purged connection. The helium was used as carrier gas (3 mL/min for 12 min, then ramped to 5 mL/min at 0.5 mL/min^2).The oven temperature was programmed from 80ºC (1 min) to 180ºC at 15ºC/min, then to 230ºC at 7ºC/min, then to 365ºC (4 min) at 10ºC/min. The peak identification was done by comparison of the retention times with a reference material. The amount of glycerin in each fat waste was calculated with the calibration function derived from the glycerin calibration curve. In the same way, the amount of mono-glycerides, di-glycerides and tri-glycerides were determined from the monoolein, diolein, and triolein calibration functions, respectively.

3.1.10 – Free fatty acids content

The analysis of the free fatty acids content of fat wastes was done by automatic titration (TIM 86J Titration Manager). 0.5 – 0.1 g of each fat waste were dissolved in 30 mL isopropanol

(Sigma) and titrated with NaOH (0.1M, Eka) in isopropanol, previously prepared. Three electrodes were used for titration, as mentioned in standard NF.EN.ISO 660–1999. The electrodes were positioned in the sample and, after allowing 30 s for pH reading to become stable, the titration ran at 550 rpm. The results were expressed as acidity (% oleic acid) as described by Equation 4:

$$FFAs\ (\%) = V_{titr} \times C_{titr} \times M \times \frac{100}{1000} \times w_{smp}\ (4)$$

where V_{titr} is total volume of titrant used (mL), C_{titr} is the concentration of titrant (mol/L), M is the molar weight of oleic acid and W_{smp} is the fat waste weight (g).

3.1.11 – Fatty acids profile analysis

The fatty acids composition of fat wastes was determined by gas chromatography (GC). Accurately weighed (~25 mg) fat wastes were mixed with 2 mL of methanol (Fisher) containing 5% (v/v) of acetyl chloride (Sigma) in 10 mL capped vials. The mixtures were heated at 80ºC for 60 min. After cooling to room temperature, 1 mL of deionized water was added to each sample and the mixtures were stirred in the vortex (2500 rpm, 30 s). Two layers were obtained. 600 µL of the upper organic phase were transferred into another vial and mixed with 400 µL of methylheptadecanoate (Sigma) at a concentration of 10 mg/mL. Samples were injected (0.5 µL) on a Thermo Scientific TRACE GC Ultra equipped with a PTV inlet with backflush option and a flame ionisation detector (FID), automated by a TriPlus Autosampler for liquids. A polar Thermo Scientific TRACE TR-BIODIESEL column (30 m, 0.25 mm, 0.25 µm) and a pre-column (2 m, 0.53 mm) were used. Helium was used as the carrier gas (2 mL/min, constant flow mode). The oven temperature was programmed from 120ºC (0.5 min) to 220ºC (1 min) at 30ºC/min, then to 250ºC (5 min) at 10ºC/min. The PTV injector program consisted of: 90ºC to 260ºC (10ºC/s), split flow 100 mL/min; transfer time = 3 min; cleaning: 360ºC, split 250 mL/min x 20 min. Peak identification was done by comparison of the retention times with a reference material. The ester contents were determined as showed in Equation 5:

$$\%\ Ester = \frac{(\frac{AP}{API})}{\sum(\frac{AP}{API})} \times 100\%\ (5)$$

where AP is the peak areas ranging from C14:0 to C18:3, and API is the peak area of methylheptadecanoate.

3.2 – Microbial cultivation experiments

3.2.1 – Bacterial strains

Bacterial cultures, preserved by liophilization, of *Pseudomonas oleovorans* NRRL B-14682, *Pseudomonas oleovorans* NRRL B-14683, *Pseudomonas oleovorans* NRRL B-778, *Pseudomonas oleovorans* NRRL B-3429, *P. resinovorans* NRRL B-2649, *P. resinovorans* NRRL B-4205, *P. citronellolis* NRRL B-2504, *P. stutezeri* NRRL B-775, *P. stutezeri* NRRL B-2461 and *C. testosteroni* NRRL B-2611 were offered by the National Center for Agricultural Utilization Research, USA. *C. necator* DSM 428 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

All bacterial strains were reactivated by inoculation of the lyophilized cells in liquid Luria–Bertani LB (20 mL) in 100mL shake flasks, with cotton filters. Cells grew aerobically for 48 h, at 30°C and at 200 rpm, on a orbital shaker. The cultures were transferred to sterile 2 mL cryovials containing sterile 20% glycerol (Sigma) as a cryoprotectant agent. The vials were stored at -80°C. For short term storage (up to 24 h), the cultures were streaked onto solid LB agar plates and incubated at 30ºC until isolated colonies were clearly observed. The plates were then kept at 4°C.

3.3.2 – Media

LB liquid medium was used for pre-cultivation of bacteria. LB composition was as follows (per litre of deionized water): bactotriptone 10.0 g (Quilaban), yeast extract 5.0 g (Panreac) and NaCl 10.0 g (Panreac). The pH was readjusted at 7.0 with 5 M NaOH and then LB medium was sterilized in a steam autoclave (Uniclave 77) at 120ºC for 20 min. Solid LB medium was prepared by adding Agar (15 g/L, Pronagar). The mineral medium for bacterial cultivation in shake flasks and bioreactor experiments had the following composition (per liter): $(NH_4)_2HPO_4$ 1.1 g (Scharlau), K_2HPO_4 5.8 g (Panreac), KH_2PO_4 3.7 g (Panreac), 1 mL $MgSO_4.7H_2O$ (CMD Chemicals) and 1.0 mL micro-elements solution. Micro-elements solution had the following composition (in 1M HCl, Sigma): $FeSO_4.7H_2O$ 2.78, $MnCl_2.4H_2O$ 1.98, $CoSO_4.7H_2O$ 2.81, $CuCl_2.2H_2O$ 0.17, $ZnSO_4.7H_2O$ 0.29, $CaCl_2.2H_2O$ 1.67. Before sterilization at 120ºC and 20 min, mineral medium pH was readjusted at 7.4 with 5 M NaOH. $MgSO_4.7H_2O$ and micro-elements solution were prepared and autoclaved separately and added aseptically to the medium after cooling. The substrate (fat wastes) was sterilized separately and added while hot (~50ºC) to the mineral medium, under aseptic conditions, to give an initial concentration of 20 g/L of fat wastes.

3.2.3 – Shake flask experiments

For preparation of inocula, an isolated colony was taken from an agar plate prepared as described above and inoculated into 50 mL liquid LB, in 250 mL shake flasks. Cells were incubated at 30ºC and 200 rpm, for 72 h.

The cultures thus obtained were used as inocula for the shake flask cultivations in 250 mL flasks with 100 mL mineral medium supplemented with the substrate, as described above. The flasks were placed in an orbital shaker at 30ºC and 200 rpm, for 72 h, to promote the emulsification of the mixture prior to inoculation of the cultures. The pre-grown cells (10% v/v) were transferred into the mineral medium and cultivated under the same conditions for 72 h. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) (Elios α, ThermoSpectronic). pH was also measured during the experiments. At the end of experiments, 20 mL samples were analyzed for biomass and PHA quantification. The analyses were performed in duplicate.

For the preliminary culture screening study, fat wastes (margarine and refinery wastes and sludge) and virgin oil were tested as carbon sources, using *C. necator* DSM 428 . Also margarine waste was saponified (NaOH 5M was added to 40 mL margarine waste, at 55 ºC with stirring, until a pH of 7.08 was reached) and tested with this culture.

The other bacterial cultures described in section 3.2.1 were tested for growth and PHA accumulation using margarine waste as the sole carbon source.

3.2.4 – Bioreactor cultivation

3.2.4.1 – General cultivation conditions

Batch and fed-batch cultivations were performed in a 5 L bioreactor (BioStat® B-Plus, Sartorius) with a double jacketed glass vessel and a maximum working volume of 5 L. The tank geometry was an unbafled cylindrical tank equipped with a Rushton turbine.

In all experiments, the temperature and the pH were kept at 30± 0.1ºC and 7.0 ± 0.1, respectively. pH was controlled by the automatic addition of 5 M NaOH or NH_4OH solutions (25%, Scharlau) and 2M HCl solutions. The aeration was provided by compressed air spargers placed at the bottom of the vessel, with constant aeration rate (1 vvm). The dissolved oxygen (DO) concentration was monitored with a DO electrode (Mettler Toledo) and maintained at 30% of air saturation by automatically adjusting the stirring speed between 300 and 800 rpm and supplementation with pure oxygen. Antifoam (Sigma) was automatically added as anti-foam agent. The batch and fed-batch culture schemes are shown in Figure 3.1. During the experiments, cell growth was monitored by measuring the OD_{600}.

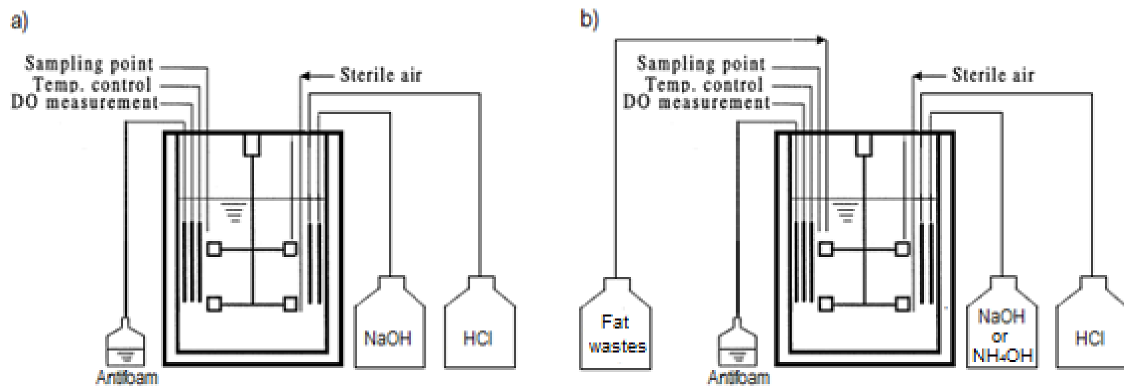

Figure 3.1 – Experimental setup of batch (a) and fed-batch (b) culture systems.

3.2.4.2 – Batch cultivation experiments with selected bacterial strains

The selected strains, *C. necator* DSM 428, *Pseudomonas oleovorans* NRRL B-14683, *P. citronellolis* NRRL B-2504 and *C. testosteroni* NRRL B-2611, were cultivated in the bioreactor under a batch mode. For inocula preparation, cells (40 mL) grown in LB for 24 h at 30°C on a orbital shaker (200 rpm) were inoculated into 400 mL of mineral medium containing 2% (wt/v) of fat wastes (margarine waste) and incubated for 72 h under the same conditions. This culture was inoculated into the bioreactor with an initial volume of 4 L. NaOH (5 M) was used to control the pH. 25 mL samples were periodically taken for quantification of cell dry weight, PHA and margarine waste concentration.

3.2.4.3 – Fed-Batch experiments with *C. necator*

C. necator DSM 428 was used in the fed-batch experiments. Two experiments were performed, using different feeding strategies during the fed-batch phase:

a) Experiment 1: after 12 h of cultivation, margarine waste pulses (~20 g/L) were based on the raise of the DO. pH was controlled by NH_4OH solution (25%) and switched to 5 M NaOH, after 19.6 h.

b) Experiment 2: after 19 h of cultivation, margarine waste pulses (~7.5 g/L) were added every 2 h. pH control was done with NH_4OH solution (25%) throughout the entire cultivation. In this case, cells grew at 24 h in inocula preparation.

In all experiments, 25 mL samples were periodically taken for quantification of cell dry weight, PHA and margarine waste concentration.

3.3 – Analytical techniques

3.3.1 – Cell dry weight and substrate quantification

The cell dry weight (CDW) and the concentration of fat wastes in the samples were both determined by gravimetry. However, due to the nature of the substrates used in the experiments, different procedures were tested to select for the most effective quantification method. For the first procedure, 20 mL of cultivation broth were mixed with the same volume of hexane (or ethanol for sludge), stirred in the vortex (2500 rpm, 30 s) and centrifuged (16743 g, 10 min). Then, the pellet was washed with 20 mL hexane and, finally, with 20 mL deionized water. The washed cell pellet was transferred to a pre-weighed plastic tube and frozen in liquid nitrogen before lyophilized for about 24 h (temperature: -42 to -45ºC; pressure less than 0.35 mbar). The CDW was determined by Equation 6:

$$CDW\ (g/L) = \frac{m_1 - m_2}{V} \quad (6)$$

where m_1 is the mass of tube with dried biomass (g), m_2 is the mass of the empty tube (g) and V is the volume of cultivation broth sample (L).

The supernatant was recovered to another pre-weighed tube and dried until complete evaporation of hexane (at room temperature, in a hood fume) and then re-weighed to determine the margarine waste concentration (Equation 7):

$$[Residual\ margarine\ waste]\ (g/L) = \frac{m_{ms1} - m_{ms2}}{V} \quad (7)$$

where m_{ms1} is the mass of tube with dried margarine waste (g), m_{ms2} is the mass of the empty tube (g) and V is the volume of cultivation broth sample (L).

For the second procedure, 10 mL of cultivation broth were lyophilized for 48 h (temperature: -42 to -45ºC; pressure less than 0.35 mbar). The dried broth was washed twice with 10 mL hexane and deionized water. Finally, the washed cell pellets were resuspended in deionized water, transferred to pre-weighed tubes and lyophilized (24 h, temperature: -42 to -45ºC; pressure less than 0.35 mbar) and reweighed. The CDW was determined by Equation 6. For quantification of the margarine waste, the lyophilized broth (5 mL) was mixed with 5 mL of hexane, vortexed (2500 rpm, 30 s) and centrifuged 10 min at 16743 g and 20°C. Subsequently, 3 mL of the upper hexane layer were transferred to a pre-weighed plastic flask and left to dry in the fume hood (at room temperature) until a constant weight was obtained. The residual fat remaining on the flask was weighed and its concentration was calculated by Equation 7. Duplicate analyses were performed.

3.3.2 – Microscopic visualization of bacterial cells and intracellular PHA granules

Cells were visualized under the optical microscope in phase contrast (Olympus BX51), using a 100x magnification. For detection of PHA granules in bacterial cells, 1 mL of cultivation broth was centrifuged at 16743 g for 5 min and the pellet was washed and centrifuged again. After suspension of the cells in deionized water and addition of 500 µL of Nile Blue A solution (Sigma), the cells were incubated at 70ºC for 10 min. Cells were put in a slide and observed by epyfluorescence light in the same microscope.

3.3.3 – PHA polymer analysis

The polymer content in the bacterial cells and its composition were determined according to the method of Braunegg et al. (1978), with small modifications. Approximately 3–5 mg lyophilized cells obtained as described above (section 3.3.1) were transferred into a screw-capped tube and subjected to methanolysis with 1 mL chloroform (Sigma) containing 1 mg/mL methylbenzoate (Sigma) as internal standard and 1 mL of 20% sulphuric acid in methanol, at 100°C for four hours, to break up the PHA and convert the constituents to their methyl esters. After cooling to ambient temperature, 1 mL of deionized water was added to the reaction mixture and stirred in the vortex (2500 rpm, 30 s). After phase separation, the organic phase (1 mL) was transferred to a vial containing molecular sieves (0.3nm, Merck). 1 µL of samples were analyzed in GC, on a Varian CP-3800 system equipped with an ionization flame detector and injector, at 240ºC. A ZB-5MS column (15 m, 0.25 mm, 0.25 µm) was used. Helium was used as the carrier gas with constant flow rate of 1.5 ml/min. The temperatures program for P(3HB) analysis was: the initial temperature was 60°C for 1 min; 60ºC to 120ºC (3ºC/min), 120 to 300ºC (20ºC/min). P(3HB-co-HV) copolymer (Sigma) and mcl-PHA with 3-hydroxyhexanoate (HHx) and 3-hydroxyoctanoate (HO) monomers (supplied by Biopolis) were used as standards. The polymer standards were dissolved in chloroform and treated in the same way as the samples. Quantitative determinations were done by assignment of the relative retention times of peaks to the respective 3-hydroxy fatty acid methylesters. Duplicate analyses were performed. PHA content, polymer concentration ([PHA], g/L) and active biomass concentration (X, g/L) were then calculated by Equation 8, 9 and 10, respectively.

$$\% \, PHA = \frac{\mathrm{m_{PHA}}}{\mathrm{m_{cells}}} \times 100\% (8)$$

$$[PHA] = \frac{\%\mathrm{PHA}}{100} \times \mathrm{CDW} (9)$$

$$X = \mathrm{CDW} - [\mathrm{PHA}] (10)$$

Where m_{PHA} is the amount of PHA (mg), m_{cells} (mg) is the amount of liophylized biomass in sample.

3.4 – Calculations

The specific growth rate was calculated as the increase in cell mass per unit of time. A slope linear regression between Ln (X) and time was done to determine the maximum specific growth rates (μ_{max}, h^{-1}). The growth and storage yields ($Y_{X/s}$ (g_X/g_s) and $Y_{PHA/s}$ (g_{PHA}/g_s), respectively) were determined using Equations 11 and 12, respectively:

$$Y_{X/s} = \frac{m_X}{m_s} \quad (11)$$

$$Y_{PHA/s} = \frac{m_{PHA}}{m_s} \quad (12)$$

where m_{PHA} is the mass of polymer and m_s is the mass of consumed margarine waste and m_X is the mass of liophylized biomass. The maximum PHA amount produced per liter reactor volume per unit time was defined as the volumetric productivity (r_{PHA}, g_{PHA}/L.h) (Equation 13). The specific productivity was defined as the maximum PHA amount (m_{PHAmax}) per maximum active biomass (m_{Xmax}) per time (q_{PHA}, g_{PHA}/g_X.h), (Equation 14).

$$r_{PHA} = \frac{m_{PHA_{max}}}{V.t} \quad (13)$$

$$q_{PHA} = \frac{m_{PHA_{max}}}{m_{X_{max}}.t} \quad (14)$$

The global substrate uptake rate (r_s, g_s/L.h) was determined by Equation 15:

$$r_s = \frac{m_{si} - m_{sf}}{V.t} \quad (15)$$

where m_{si} (g) is the mass of the initial margarine waste, m_{sf} (g) is the mass of the final margarine waste, V (mL) is the reactor volume and t (h) is the time of cultivation.

3.5 – Polymer extraction

Lyophilized cells obtained as described above (section 3.3.1) were extracted with chloroform (0.1 g/L) at 37 °C for 72 h, at 200 rpm on an orbital shaker. The cellular debris were removed by filtration with syringe filters with a pore size of 0.45 µm (GxF, GHP membrane, PALL) and the PHA was precipitated in ice-cold ethanol (chloroform/ethanol 1:10). Thereof, the mixture was centrifuged at 16 743 g rpm for 15 min at 4°C. The white precipitate was then recovered in a pre-weighed flask and left at ambient temperature in a fume hood for the remaining solvent to evaporate, resulting in highly pure PHA samples. The polymer content of

shake flask experiments was calculated by a gravimetric method after each extraction pretreatment. The PHA content (%) was expressed as:

$$\% PHA = \frac{m_{film}}{m_{cells}} \times 100\% (16)$$

where m_{film} (g) is the mass of the polymer film obtained after extraction from a cell mass m_{cells} (g).

3.6 – Thermal analysis

Thermal analysis was performed by differential scanning calorimetry (DSC) with a DSC Q2000 from TA Instruments interfaced with a cooling accessory (RCS). The DSC runs covered a temperature range from -90 to 200ºC with heating and cooling rate of 10 ºC/min. A small amount of samples (2-3 mg) were placed in an aluminium hermetic pan. Measurements were performed under dry high-purity nitrogen gas (at flow rate of 50 mL/min). The baseline was calibrated scanning the temperature range of the experiments with two empty pans. Calibration was carried out using Indium for temperature transitions and the heats of fusion. In this study, the glass transition temperature (T_g) was taken as the midpoint of the step-transition and the melting temperature (T_m) was also determined. The instrument was calibrated with high purity indium. The crystallinity (χ_c) of the PHA samples (Equation 17) was determined by comparing the area of the melting peak (ΔH_f) with the melting enthalpy of 100% crystalline P(3HB) ($\Delta H_{f100\%}$). An estimate of the heat of fusion of an infinite crystal of P(3HB) was estimate as 146 J/g (Barham et al., 1984).

$$\chi_c = \frac{\Delta H_f}{\Delta H_{f100\%}} \times 100\% (17)$$

4 Results and Discussion

4.1 – Fat wastes characterization

Two lots of the three fat waste materials were supplied by FIMA SA – Unilever. The physical and chemical characteristics of all fat wastes were determined. As shown in Figure 4.1, the visual appearance of margarine (a) and refinery (b) wastes was very similar: both were solid at ambient temperature and were apparently homogeneous. However, the margarine waste had a yellowish colour, while the refinery waste was darker and brownish. In contrast, the sludge (c) showed two phases, a solid phase that contained a large amount of solid particles, and a liquid phase, making this sample very heterogeneous. These characteristics are probably related to their origin in the manufacturing process, which was described in section 2.1.

Figure 4.1 – Fat wastes from the manufacture process of margarine: a) margarine waste; b) refinery waste; c) sludge.

The physical and chemical characterization of the fat wastes included the determination of: density, pH, water content, solubility in water and organic solvents, and composition in organic and inorganic compounds. The pH of all fat wastes was low (Table 4.1). The margarine waste had average pH of 3.97 and 4.03, for lots 1 and 2, respectively, while the refinery waste pH was significantly lower (1.84 and 1.75, for lots 1 and 2, respectively). The sludge pH was 4.35 and 4.85, for lots 1 and 2, respectively. The amount of water was not above of 9% in the margarine and refinery wastes and their density values were approximately 0.92 g/cm^3 in both lots (Table 4.1). These two fat wastes resulted from the initial treatment of wastewater arising from the refinery and margarine production processes, consisting practically of fats that are not soluble in water. Hence, their water content was expected to be low. Also, it is described that fats have lower densities than water (Strayer et al., 2006). On the other hand, high water contents were detected in the two sludge lots (88 and 78%, respectively) and their densities

were 0.43 and 1.04 g/cm³ for lots 1 and 2, respectively (Table 4.1). Such wastes are a mixture of all effluents coming from successive washing processes (Figure 2.2, section 2.1), resulting in high water contents, high heterogeneity and high variability between lots.

The solubility behaviour of the fat wastes was investigated in water and organic solvents (ethanol, acetone and hexane) and the results are presented in Table 4.1. Those organic solvents were chosen for being the most used in oil extraction processes (Johnson & Lusas, 1983; Sivarao et al., 2012). Regarding the margarine waste, the highest solubility was obtained in hexane (93.05 and 97.50%, for lots 1 and 2, respectively) and acetone (95.18 and 99.14%, for lots 1 and 2, respectively). Also, hexane had the best solubility values for the refinery wastes (96.83 and 95.97%, for lots 1 and 2, respectively) (Table 4.1). As expected, the solubility of these two wastes in water was very low, as shown in Table 4.1. For the sludge, only the organic solvents were tested, because that waste material had a large amount of water. The solubility results obtained were significantly different among lots 1 and 2 (Table 4.1). In lot 1, acetone and ethanol solubilised close to 80% of the material, while only 14.67% was solubilised using hexane. In lot 2, the solubility in hexane was higher (45.93%), while the solubility in acetone and ethanol decreased to 44.47 and 52.53%, respectively. The heterogeneity of the sludge explains these differing values between the two analysed lots.

Table 4.1 – Physical properties of fat wastes. The values of pH, density and water content are shown ± SD (n=2).

	Substrate	pH	Density (g/cm³)	Water content (%)	Solubility (%)			
					Hexane	Acetone	Ethanol	Water
Lot 1	Margarine	3.97±0.7	0.92±0.0	8.90±0.4	93.05	95.18	84.58	0.53
	Refinery	1.84±0.0	0.92±0.0	7.18±0.1	96.83	89.29	85.97	2.87
	Sludge	4.35±0.1	0.43±0.0	87.55±0.3	14.67	80.68	79.74	-
Lot 2	Margarine	4.03±0.3	0.91±0.0	4.70±1.0	97.50	99.14	91.38	0.11
	Refinery	1.75±0.0	0.92±0.0	1.11±0.2	95.97	90.87	88.00	0.28
	Sludge	4.85±0.0	1.04±0.0	77.92±1.6	45.93	44.47	52.53	-

Considering that bacteria cultivation experiments are performed in aqueous media, the ability to form stable fat wastes/water mixtures was tested for all fat wastes. Some factors influence the miscibility of fats in water, like temperature, stirring and the addition of emulsifiers (Paasimaa, 2005). Although the margarine and refinery wastes had low water solubility (Table 4.1), when the aqueous mixtures were kept at 30ºC, under constant stirring (200 rpm) for three days, homogeneous emulsions were formed (Figure 4.2a and 4.2b). Also, after being left at ambient temperature for a week, the aqueous mixtures maintained their homogeneity, without phase separation, thus revealing a good stability. In spite of both fat wastes showing a homogeneous cream coloured phase, the refinery waste aqueous mixtures had some suspended particles that could be fat aggregates (Tamilvanan, 2004). The emulsifiers used during the margarine production process may still have been present in the margarine wastes

and sludge and have provided emulsion stability. The overflow between effluents may explain the presence of emulsifiers in refinery waste and also provided the emulsion (Figure 2.2, section 2.1). Due to their heterogeneity, when the sludge samples were added to water, many solid particles remained in suspension or got stuck on the inner wall of the flask (Figure 4.2c).

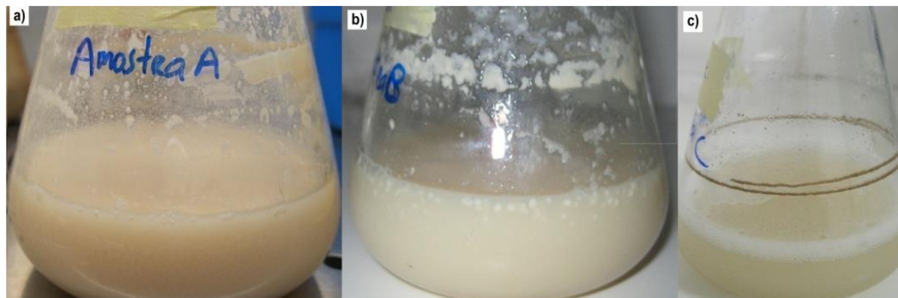

Figure 4.2 – Mineral medium supplemented with fat wastes: a) margarine; b) refinery; c) sludge after being kept in a rotary shaker at 200 rpm and 30ºC for 72 h.

The elemental analysis composition of each fat waste is presented in Table 4.2. The main component present in all fat wastes was carbon, with average contents of 75, 74 and 67% for margarine and refinery wastes and sludge, respectively. 10-13% hydrogen contents were observed in all fat wastes. The presence of carbon and hydrogen atoms is due to the long hydrocarbon chains of the fatty acids (Strayer et al., 2006). No nitrogen was detected in the margarine and refinery wastes, in either lot, while small amounts of nitrogen were detected in the sludge samples (Table 4.2). Trace amounts of sulphur were observed only in the margarine waste (lot 1) and sludge (lot 2). The presence of this element may be related to the use of sulphuric acid in fatty acid regeneration and the subsequent overflow between effluents (Figure 2.2, section 2.1).

The total sugar content was also quantified for all fat wastes (Table 4.2). Low sugar contents were measured: 11.27 and 10.12% for margarine, 6.80 and 10.92% for refinery, 1.41 and 5.93% for sludge, in lots 1 and 2, respectively. The contact of effluents from vegetable oil processing with other effluents might explain the presence of sugars in these wastes.

Table 4.2 – Chemical properties of fat wastes: elemental analysis (Carbon – C; Nitrogen – N; Hydrogen – H; Sulphur – S) and total sugar. The values of elemental composition and sugar content are shown ± SD (n=2).

	Substrate	Elemental composition (%)				Total sugar (%)
		C	N	H	S	
Lot 1	Margarine	75.25 ±0.5	0	12.43 ±0.3	0.39 ±0.1	11.27 ±3.3
	Refinery	74.85 ±0.0	0	12.53 ±0.0	0	6.80 ±0.1
	Sludge	67.23 ±0.6	1.3 ±0.0	10.89 ±0.3	0	1.41 ±0.0
Lot 2	Margarine	74.94 ±0.0	0	11.81 ±0.2	0	10.12 ±1.8
	Refinery	73.53 ±0.3	0	12.48 ±0.1	0	10.92 ±0.3
	Sludge	66.38 ±0.4	1.92 ±0.0	11.19 ±0.2	0.13 ±0.0	5.93 ±1.1

According to the ICP analysis, aluminium, calcium, iron, sodium, phosphorus and potassium were detected in the fat wastes, in both lots, but their total contents were low (0.20–3.33 g/L) (Table 4.3). Moreover the two lots of margarine and refinery wastes had rather different inorganic content and composition. While in lot 1, the total inorganic content was 3.33 and 1.59 g/L, respectively, in lot 2 their content was only 0.55 and 0.20 g/L, respectively (Table 4.3). Regarding the margarine waste, the major inorganic components were calcium and iron (Table 4.3). In the refinery waste, iron had the highest content (Table 4.3). It was observed a similar inorganic content in sludge between lot 1 and lot 2 (0. 88 and 0.99 g/L, respectively), as shows Table 4.3. Only calcium and phosphorus achieved concentrations above 0.15 g/L in sludge for both lots (Table 4.3). Vegetable oils contain varying levels of trace elements depending upon exposure to different compounds during plant cultivation season or during the extraction and processing of oils, such as bleaching, refining, and deodorization. In addition, they can be incorporated by contamination from the metal processing equipment and thus be suspended in the oil (Lepri et al., 2011). These aspects might explain the presence of trace elements in fat wastes and the differences between lots.

Table 4.3 – Trace elements presented in fat wastes: aluminium (Al), calcium (Ca), iron (Fe), sodium (Na), phosphorus (P) and potassium (K), determined by ICP.

	Substrate	Mineral salts (mg/L)							Total (g/L)
		Al	Ca	Mg	Fe	Na	P	K	
Lot 1	Margarine	0.08	1.52	0.04	1.41	0.12	0.13	0.03	3.33
	Refinery	0.05	0.12	0.01	1.02	0.12	0.23	0.03	1.59
	Sludge	0.04	0.18	0.02	0.09	0.27	0.24	0.05	0.88
Lot 2	Margarine	0.02	0.42	0.00	0.05	0.01	0.02	0.03	0.55
	Refinery	0.02	0.07	0.00	0.01	0.02	0.04	0.04	0.20
	Sludge	0.12	0.40	0.03	0.06	0.07	0.20	0.10	0.99

Figure 4.3 shows the results of the lipid profile of each fat wastes. High FFAs contents were found in all three wastes. The highest FFAs content (approximately 76%) was obtained for the margarine waste, while the other wastes had lower FFAs contents (56 and 36% in the refinery waste and sludge, respectively) (Figure 4.3). Margarine waste had high FFAs content, because these wastes had mainly hydrolyzed oils not used in refinery process (described in section 2.1). On the other hand, soapstock from neutralization and interestification processes were constituted by FFAs combined with the alkaline catalyst, as described in section 2.1. When this soapstock was subjected to the regeneration process, refinery wastes were produced with high FFAs content. Less than 21 and 12% tri- and diglycerides were found in the margarine and refinery wastes, respectively (Figure 4.3). Trace amount of monoglycerides were detected and no glycerol content was observed. Such tri-, di- and monoglycerides might have been introduced in these wastes during oil extraction. The sludge samples only presented FFAs (Figure 4.3). The other components were probably removed during the wastewater treatment process.

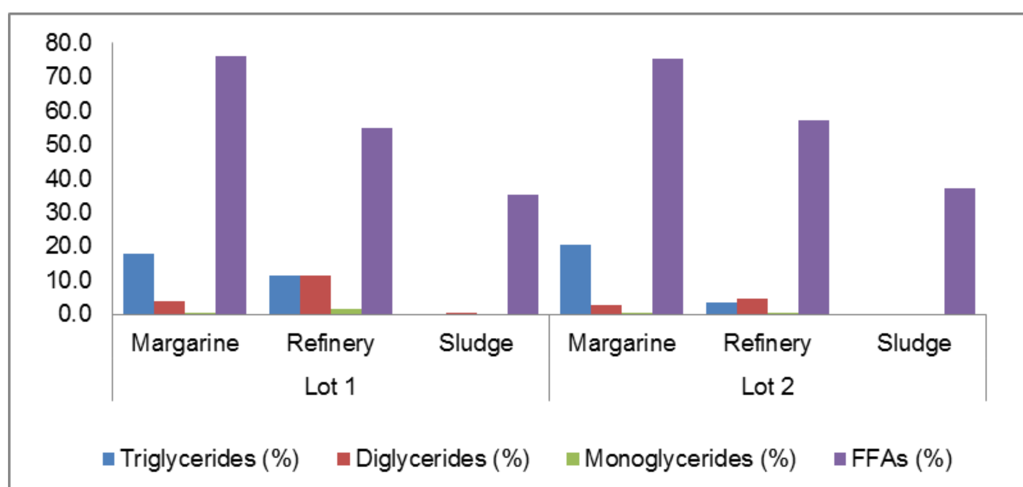

Figure 4.3 – Lipid profile (%) of fat wastes: triglycerides (blue), diglycerides (red), monoglycerides (green) and FFAs (purple).

The fatty acid profile in the fat wastes was determined with a methanolysis assay (Table 4.4). Oleic (37–54%), linoleic (4–27%), stearic (11–18%) and mystiric acids (4–14%) were the predominant components detected in all wastes. The fatty acid profile for the two margarine waste lots were identical, except for linolenic acid (27.01% in lot 1 and 21.61% in lot 2) and oleic acid, which had a lower content (48.67%) in lot 1 than in lot 2 (54.28%). The refinery waste also showed similar fatty acid distribution between lots, but the content in mystiric (4.83% in lot 1 and 12.80% in lot 2) and oleic acids (48.64% in lot 1 and 37.19% in lot 2) were slightly different. In contrast, the sludge had more significant differences between the two analysed lots: oleic acid was the main component in both lots (41.26 and 54.05%), but its content was higher in lot 2; a high content of mystiric acid (14.05%) was detected in lot 1, but its content was lower (7.81%) in lot 2, while the opposite was observed for linoleic acid (4.89% in lot 1 and 16.43% in lot 2). Vegetable oils used in the margarine production manufacture contained similar fatty acids

composition, where oleic and linoleic acids had the highest percentage (Zambiazi et al., 2007). The percentage of the total saturated fatty acid content was about 21 to 36% and because of that fat wastes were highly viscous and mostly in solid form at ambient temperature.

Table 4.4 – The fatty acids distribution in fat wastes for lots 1 and 2.

	Substrate	Mystiric (%)	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)
Lot 1	Margarine	6.20	3.33	11.50	48.67	27.01	3.29
	Refinery	4.83	2.82	17.41	48.64	24.55	1.75
	Sludge	14.05	6.41	11.69	41.26	4.89	2.68
Lot 2	Margarine	6.20	3.22	12.40	54.28	21.61	2.28
	Refinery	12.80	5.21	18.34	37.19	23.76	2.70
	Sludge	7.81	3.72	12.07	54.05	16.43	3.45

Since the margarine and refinery wastes were composed primarily of long-chain acids with 12 to 22 carbons, these wastes were strongly hydrophobic. Therefore, the low pH values are probably related to the large amount of fatty acids present in the fat wastes. Due to their nature, fatty acids are easily extracted with nonpolar solvents, but they are poorly soluble in water, which explains the low percentage of water extraction (Strayer et al., 2006). The sludge had high water content and it fatty acids content was lower than other wastes, which may explain the poor solubility in hexane. Hexane is constituted by six carbon atoms and is the most nonpolar of the solvents used. Thereby, it has more affinity for hydrophobic compounds like fatty acids. While ethanol and acetone have, respectively, two and four carbon atoms, they are slightly more polar and can solubilise easier in water than hexane (Sivarao et al., 2012).

The main components presented in margarine and refinery wastes were FFAs, tri-, diglycerides and sugar (Figure 4.4). The sludge contained basically water, FFAs and sugar.

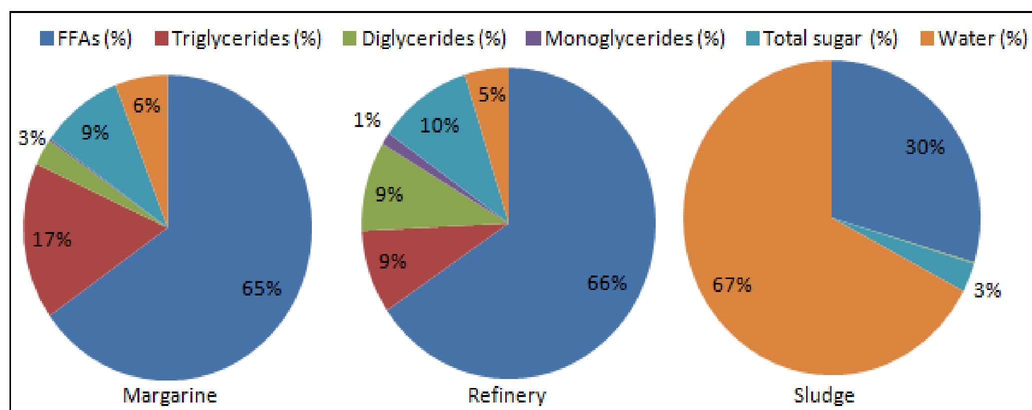

Figure 4.4 – The main components (%) in fat wastes: FFAs (blue) triglycerides (red), diglycerides (green), monoglycerides (purple), total sugar (light blue) and water (orange).

For most parameters determined (pH, density, water content and elemental composition) the margarine and refinery wastes had very similar values among replicas (low standard deviation values), indicating little variability among lots. On the other hand, for the sludge samples, there were significant differences between replicate analyses and the two lots, which reflect their heterogeneity. Moreover, the margarine and refinery wastes resulting from the margarine production and refinery processes apparently have stable physical and chemical characteristics, because the two lots analyzed did not present many differences in all parameters.

According to the fat wastes characterization, these materials attract great interest as carbon source for microbial cultivation due to their high carbon content, mainly FFAs with minor sugars content. However, the presence of linolenic acid may affect the cell growth as it has been reported as a cell growth inhibitor (Kahar et al., 2004). It was revealed that the low pH of fat wastes did not affect the bacteria cultivation, due to the addition of base to raise the pH and the buffering capacity of the mineral medium. Although fat wastes are insoluble in water, homogeneous emulsions were obtained in the aqueous mixtures. This fact is important to ensure that growth was not impaired by an inadequate supply of nutrients and oxygen (Doran, 1995). On the other hand, hexane and ethanol was revealed as the best solvent to extract and quantify fat wastes.

4.2 – Shake flask experiments

In order to evaluate the best PHA producer strain, several bacteria were selected for screening experiments, based on their ability to produce PHA from fats and oils reported in the literature (Table 2.3, section 2.4).

Initially, the three fat-containing wastes supplied by FIMA were screened for their suitability to support bacterial cell growth and PHA synthesis. *C. necator* DSM 428 was selected for the substrate screening because it was reported to be able to grow and accumulate high PHA contents in the biomass in the presence of an ample range of carbon sources, including long-chain fatty acids (Budde et al., 2011a; Fukui et al., 1998; Kahar et al., 2004; Kamilah et al., 2013; Kocer et al., 2003; López-Cuellar et al., 2011; Ng et al., 2010; Park & Kim, 2011; Taniguchi et al., 2003; Verlinden et al., 2011) (Table 2.3, section 2.4).

As shown in Table 4.5, *C. necator* was able to grow and accumulate PHA in mineral medium supplemented with all three substrates as sole carbon sources. However, after 72 h of cultivation, cell growth was considerably lower with the refinery waste (1.7 g/L) than with the margarine waste (10.4 g/L), even though high PHA contents were obtained in both assays (58.8 and 69.4% (wt/wt), respectively) (Table 4.5). The CDW achieved in the sludge experiment (5.2 g/L) was not accurate, because it was not possible to remove all the solid particles during the cell washing process. The CDW and polymer content obtained with the margarine waste were higher than the values reported by Verlinden et al. (2011) (3.7 g/L and 32% (wt/wt), respectively) for *C. necator* grown on 20 g/L waste rapeseed oil, in similar cultivation conditions.

Taniguchi and co-workers (2003) reported that *C. necator* accumulated a PHA content in the biomass of 63% (wt/wt), using waste oils in shake flask experiment. However, when tallow was used as carbon source, they obtained a higher PHA content (80%). On the other hand, Fukui & Doi (1998), reported higher PHA content (82%, wt/wt), but lower CDW (4.1 g/L) for *C. necator* grown on 10 g/L oleic acid.

Due to its high FFAs content compared with other carbon sources (Figure 4.3), the margarine waste not only demonstrated an efficient production of PHA, but also obtained the highest cell dry weight. For that reason, all the following experiments were done with margarine waste as substrate. Also, aiming to improve the performance of bacteria on the margarine waste, this material was saponified (Table 4.5).

Table 4.5 – Cell dry weight, PHA content and PHA composition for the shake flask experiments with different bacteria grown on different fat-rich materials (margarine, saponified margarine and refinery wastes, sludge, and virgin oil).

Bacteria	Substrate	CDW (g/L)	PHA content (%, wt/wt)	PHA composition (mol %)			
				HB	HHx	HO	HD
***C. necator* DSM 428**	Margarine	10.4	69.4	100	0	0	0
	Refinery	1.7	58.8	100	0	0	0
	Sludge	5.2	4.2	100	0	0	0
	Saponified Margarine	7.0	54.9	100	0	0	0
	Virgin oil	6.7	49.9	100	0	0	0
***C. testosteroni* NRRL B-2611**	Margarine	2.5	29.6	100	0	0	0
***P. citronellolis* NRRL B-2504**		6.3	8.2	0	21	42	37
***P. oleovorans* NRRL B-778**		0	-	-	-	-	-
***P. oleovorans* NRRL B-3429**		3.2	0.9	0	0	71	29
***P. oleovorans* NRRL B-14682**		3.7	0.8	0	0	72	28
***P. oleovorans* NRRL B-14683**		2.1	1.4	0	0	70	30
***P. resinovorans* NRRL B-2649**		3.9	0	-	-	-	-
***P. resinovorans* NRRL B-4205**		0	-	-	-	-	-
***P. stutzeri* NRRL B-775**		0	-	-	-	-	-
***P. stutzeri* NRRL B-2461**		0	-	-	-	-	-

The saponification has been reported to increase the solubility of fats in aqueous media (Karasulu et al., 2011). *C. necator* was grown on saponified margarine waste and, after 72 h, it attained a CDW of 7.0 g/L. However, the PHA content (54.9%, wt/wt) was lower than that obtained on the margarine waste (69.4%, wt/wt) (Table 4.5). PHA production by *C. necator* from saponified margarine waste was also lower when compared with emulsified plant oil (79% wt/wt) (Budde et al., 2011a).

Virgin oil products, such as soybean oil, canola oil and palm oil, have been investigated for PHA production with high polymer content in the biomass (Fukui & Doi, 1998; Kahar et al., 2004; Park & Kim, 2011). Hence, for comparison, the culture was also cultivated on virgin oil, under the same cultivation conditions. *C. necator* reached lower CDW and PHA content (6.7 g/L and 49.9% (wt/wt), respectively) than on the margarine waste (Table 4.5). Because the main component of virgin oil is triglycerides, the oil is first hydrolyzed by extracellular lipase into FFAs, which are taken up by the cells for growth and PHA synthesis. Thus, in the case of margarine waste, the carbon source was more available for growth and PHA accumulation than when virgin oil was used (Cromwick et al., 1996; Kahar et al., 2004). These results suggest that *C. necator* had preference towards FFAs than saponified fats or virgin oil as carbon sources.
The polymer produced by *C. necator* in all shake flask tests was a 3-hydroxybutyrate homopolymer, P(3HB). According to the literature, *C. necator* is able to synthesize P(3HB), but when suitable organic precursors are provided, such as propionic, valeric and pentanoic acids, it is also able to produce copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate, P (HB-co-HV) (Bhubalan et al., 2008; Lee et al., 2008; Rao et al., 2010). Also, when canola oil was used as carbon source, *C. necator* was able to synthesize mcl-PHAs (López-Cuellar et al., 2011).

Besides *C. necator*, *C. testosteroni* NRRL B-2611, *P. citronellolis* NRRL B-2504, *P. oleovorans* NRRL B-778, *P. oleovorans* NRRL B-3469, *P. oleovorans* NRRL B-14682, *P. oleovorans* NRRL B-14683, *P. resinovorans* NRRL B-2649, *P. resinovorans* NRRL B-4205, *P. stutzeri* NRRL B-775 and *P. stutzeri* NRRL B-2461 species strains were screened in shake flask assay, with margarine waste to select the best PHA producers on this waste (Table 4.5).

C. testosteroni NRRL B-2611, *P. citronellolis* NRRL B-2504, *P. oleovorans* NRRL B-3429, *P. oleovorans* NRRL B-14682, *P. oleovorans* NRRL B-14683, *P. resinovorans* NRRL B-2649 were able to use the margarine waste for cell growth. Nevertheless, the PHA content in the biomass was different for each strain. *C. testosteroni* showed a maximum CDW of 2.5 g/L, with a polymer content of 29.6% (wt/wt). Thakor et al. (2005) reported the efficiency of this culture to convert different vegetable oils to mcl-PHAs (HHx, HO, 3-hydroxydecanoate (HD), 3-hydroxydodecanoate (HHD) and 3-hydroxytetradecanoate (HTD)) up to 87.5% (wt/wt), in a shake-flask experiment. Besides that, in the assay with margarine waste, *C. testosteroni* was able to produce only P(3HB) homopolymer, maybe due to the substrate’s complexity.

On the other hand, *P. citronellolis* NRRL B-2504 cell growth was higher (6.3 g/L) than that of *C. testosteroni*, but the biomass content in PHA was much lower (8.2%, wt/wt). The polymer produced was a mcl-PHA, composed of 21 mol% HHx, 42 mol% HO and 37 mol% HD (Table 4.5). Cromwick and co-workers (1996) reported lower CDW (1.7 g/L) and lower mcl-PHA

content (3%, wt/wt) for *P. citronellolis* grown on tallow free fatty acids. The produced PHA was composed of 10 mol% HHx, 48 mol% HO, 28 mol% HD, 8 mol% HHD and 1 mol% HTD. The differences in the composition of the mcl-PHA from margarine waste and tallow free fatty acids (described by Cromwick et al. (1996)) could be related to some factors like the substrates composition in fatty acids, namely different ratios of saturated and unsattured fatty acids, the specificity of PHA-synthesizing system and the degradation of long-chain fatty acids. On the other hand, since PHA synthase has high specificity for 3-hydroxyoctanoyl-CoA and 3-hydroxydecanoyl-CoA, the mcl-PHA polymer obtained with the margarine waste had high values of HO and HD monomers (Ashby & Foglia, 1998).

All *P. oleovorans* strains tested, except *P. oleovorans* NRRL B-778, were able to grow on the margarine waste and accumulate PHA, though their CDW and the polymer content were very low (2.1–3.7 g/L and 0.8–1.4%, wt/wt, respectively). Nevertheless, the synthesized polymers were interesting since they were mcl-PHA composed of HO and HD monomers. *P. oleovorans* NRRL B-14683, *P. oleovorans* NRRL B-14682 and *P. oleovorans* NRRL B-3429 synthesized a copolymer of HO (70–72 mol%) and HD (28–30 mol%). Cromwick and co-workers (1996) also reported that up to 18% (wt/wt) of mcl-PHA (HHx, HO, HD, HHD, HTD monomers) was accumulated by *P. oleovorans* NRRL B-14683 grown on tallow free fatty acids. Interestingly, *P. oleovorans* NRRL B-14682 was reported to synthesize P(3HB) (27%, wt/wt) from soy-based biodiesel production (Ashby et al., 2004), but no mcl-PHA production from fats or oils has been reported in the literature. Besides that, production of PHA by *P. oleovorans* NRRL B-3429 was also not found in literature, making it an interesting strain.

Despite *P. resinovorans* NRRL B-2649 has grown to a CDW of 3.9 g/L, no polymer accumulation was observed (Table 4.5). The other tested *P. resinovorans* strain (NRRL B-4205), *P. stutzeri* NRRL B-775 and *P. stutzeri* NRRL B-2461 were not able to grow on the margarine waste, despite many *Pseudomonas* species have been reported as being able to grow on oil/fat containing substrates (Du et al., 2012). *P. resinovorans* NRRL B-2649 was reported to grow and accumulate mcl-PHAs using unhydrolyzed tallow and triglyceride substrates as carbon sources (Ashby & Foglia, 1998; Cromwick et al., 1996). *P. stutzeri* NRRL B-1317 was found to synthesize mcl-PHAs when growth on glucose and fatty acids (He et al., 1998). Also, mcl-PHAs were obtained from linoleic acid and methylated oils (corn oil acids and laurel seed oil acids), when *Pseudomonas oleovorans* NRRL B-778 was cultivated (Kocer et al., 2003).

Apart from the differences in cultivation medium in the different reported literature studies, the carbon source used in the shake flask experiments of this study was more complex and might explain why some of the tested bacterial strains did not grow or produce polymer. Based on the highest polymer content and/or the composition of the produced PHA, *C. necator* DSM 428, *C. testosteroni* NRRL B-2611, *P. citronellolis* NRRL B-2504, *P. oleovorans* NRRL B-14683 were chosen for subsequent experiments in bioreactor under controlled operation conditions.

4.3 – Batch bioreactor cultivation experiments with the selected bacterial strains

After the preliminary study in shake flask to evaluate the storage capacity performance of each bacterium, batch bioreactor experiments were performed with the four organisms selected using margarine waste as carbon source. The objective of these experiments was to evaluate each culture's performance under controlled cultivation conditions. All the batch cultivations were operated at the same cultivation conditions (temperature, DO, stirring and pH) with initial concentration of 20 g/L of margarine waste. The experiments were terminated when a decrease of the stirring speed and the increase of DO was observed, since it indicated that the metabolic activity of the cells had decreased. Figures 4.5, 4.7, 4.9, 4.11 and Table 4.6 show the concentration profiles for active biomass and polymer concentration, and the kinetic parameters of each batch experiment, respectively.

It was not possible to accurately quantify the substrate concentration during the assays and only the initial and final samples of each experiment were analysed with an optimized method developed during this study. The presence of saponified fats led to the formation of three-phase systems (water, hexane and saponified fats) by adding hexane to the culture broth. Due to their amphipathic nature, saponified fats were not completely removed during washing, thus leading to incorrect substrate quantification. The optimized method consisted in first removing water from the broth samples by lyophilisation (see section 3.3.1). Then, hexane was added to the dried broth and only a two-phase system occurred. After centrifugation, the supernatant contained residual margarine waste dissolved in hexane (hydrophobic phase), while the pellet had the cellular material.

Also, Nile Blue staining was used to assess biopolymer accumulation by visualization of the PHA granules under the optical microscope as orange fluorescence inside the cells (Figures 4.6, 4.8, 4.10 and 4.12).

Table 4.6 – Kinetic parameters for cultivation of *C. necator* DSM 428, *C. testosteroni* NRRL B-2611, *P. citronellolis* NRRL B-2504 and *P. oleovorans* NRRL B-14683 in batch bioreactor experiments using margarine waste as carbon source.

Bacteria	$\mu_{máx}$ (h^{-1})	CDW (g/L)	X (g/L)	PHA content (%, wt/wt)	PHA (g/L)	r_{PHA} (g_{PHA}/L.h)	q_{PHA} (g_{PHA}/g_x.h)	r_s (g_s/L.h)	$Y_{x/s}$ (g_x/g_s)	$Y_{PHA/s}$ (g_{PHA}/g_s)
***C. necator* DSM 428**	0.15	11.2	4.7	55.8	6.4	0.33	0.061	0.51	0.37	0.50
***C. testosteroni* NRRL B-2611**	0.40	14.0	13.0	7.2	1.1	0.06	0.015	0.70	0.16	0.08
***P. citronellolis* NRRL B-2504**	0.39	8.1	8.0	7.0	0.6	0.04	0.004	0.84	0.48	0.04
***P. oleovorans* NRRL B-14683**	0.37	3.4	3.4	8.5	0.3	0.02	0.004	0.70	0.21	0.02

4.3.1 – Batch cultivation experiment with *C. necator* DSM 428

Figure 4.5 shows the cultivation profile of *C. necator* on margarine waste. The exponential growth phase was initiated after a 3 h lag phase, with a maximum specific growth rate was 0.15 h^{-1} (Figure 7.1, in section 7.2) (Table 4.6.). A significant increase of the active biomass was only observed after 10 h of cultivation. Polymer production showed a similar trend soon after (~12 h). At the end of experiment (20 h), the CDW reached a maximum of 11.2 g/L with a P(3HB) content of 55.8% (wt/wt), corresponding to 6.4 g/L of polymer concentration and 4.7 g/L of active biomass concentration (Table 4.6). The volumetric and specific productivities were 0.33 g_{PHA}/L.h and and 0.061 g_{PHA}/g_X.h, respectively. From the initial 20 g/L margarine waste available, 10 g/L were consumed, which corresponded to a global substrate uptake rate of 0.51 g_s/L.h (Table 4.6). The growth and storage yields were 0.37 g_x/g_s and 0.50 g_{PHA}/g_s, respectively.

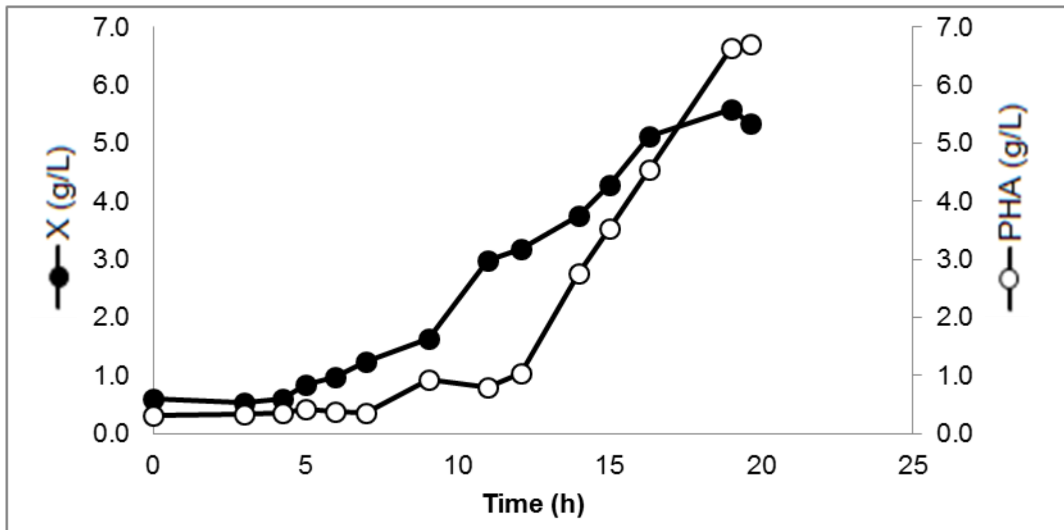

Figure 4.5 – Batch cultivation profile by *C. necator* DSM 428 using margarine waste as carbon source

The microscope photograph show that the morphology of *C. necator* cells changed over time: cells elongated as polymer granules were being accumulated (Figure 4.6a and 4.6b). The high fluorescence observed is related with the high PHA content values obtained (55.8%, wt/wt).

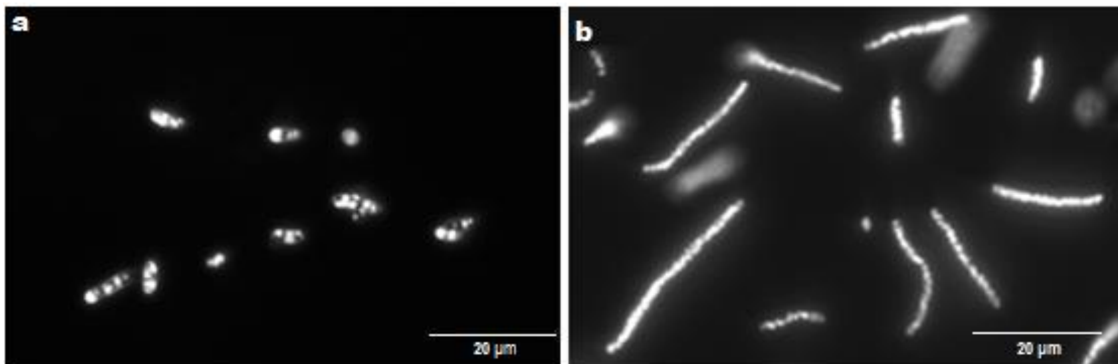

Figure 4.6 – Optical microscopic photographs (100x) of bacterial *C. necator* DSM 428 cells stained with Nile Blue at the beginning (a) and at the end (b) of the batch bioreactor experiment.

The polymer content in the biomass was lower than the value obtained in the shake flask assay (69.4%, wt/wt) (Table 4.5), but the CDW concentration was slightly higher (11.2 g/L in the bioreactor cultivation against 10.4 g/L in shake flask). Besides that, the volumetric productivity increased significantly from 0.10 g_{PHA}/L.h to 0.33 g_{PHA}/L in the batch bioreactor culture, which is probably due to the controlled conditions (pH, dissolved oxygen concentration, stirrer speed) that were kept in the bioreactor and not in the shake flasks (Doran, 1995). Similarly to the shake flasks assays (Table 4.5) the produced polymer was a HB homopolymer (Table 4.8).

Kahar et al. (2004) reported PHA production by *C. necator* cultivated on soybean oil, achieving an active biomass concentration close to 17 g/L (in 18h) and a PHA content above 30% (wt/wt). At similar time of cultivation, the PHA content obtained with margarine waste as

carbon source was higher (55.8%, wt/wt) than that reported for soybean oil, but the active biomass concentration was lower (4.7 g/L). These results indicate that *C. necator* was more efficient in accumulating than growing in the margarine waste, as shown by the differences between storage and growth yields (0.50 g_{PHA}/g_s and 0.37 g_x/g_s, respectively). The values obtained with margarine waste were also higher than that reported for jatropha oil as substrate: a range of 2-4 g/L of CDW and 40% (wt/wt) of PHA content were obtained after 12 h of cultivation, corresponding to a volumetric productivity of 0.1 g_{PHA}/L.h (Ng et al. 2010). This value is considerably lower than that obtained with margarine waste.

In this study, the culture was unable to use all the available complex substrate (only half of it was consumed). Since *C. necator* can not directly utilize try-, dy- and monoglycerides for cell growth and PHA synthesis, these materials must first be hydrolyzed into FFAs by lipases and then incorporated into the cells and metabolized (Kahar et al., 2004). Considering that the margarine waste had high FFAs content (75%), the lipase activity of the cells might have been low and the cellular mechanism was probably directed for the use FFAs and the culture was not able to use the try-, dy- and monoglycerides present in the medium. Furthermore, some fatty acids may have affected cell growth. Kahar et al. (2004) reported that *C. necator* grew well on palmitic acid, oleic acid and linoleic acid, but could not grow well on linolenic acid. Although, the margarine waste had approximately 3% of linolenic acid, the accumulation of this long-chain fatty acid in culture broth could limit the transfer of other fatty acids into the cells. Hence, the unutilized margarine waste might have inhibited cell metabolism.

Another possible explanation for the low substrate consumption and, subsequently, low biomass production could be related with the high initial PHA content (35.2%, wt/wt) of the cells. Apparently, during the first hours of the experiment (8 h), the microorganism had preference to use the accumulated intracellular polymer reserves instead of consuming the external substrate.

4.3.2 – Batch cultivation experiment with *C. testosteroni* NRRL B-2611

After *C. testosteroni* was inoculated in the bioreactor, nearly 6 h of lag phase were observed. The maximum specific growth rate was 0.40 h^{-1} (Figure 7.2, in section 7.2) (Table 4.6). Polymer production started after 8 h of cultivation, as shown in Figure 4.7. During the experiment, both polymer and active biomass concentration had a similar profile, increasing to 1.1 g/L and 13.0 g/L, respectively, within 18 h of cultivation (Table 4.6). *C. testosteroni* accumulated a maximum P(3HB) content of 7.2% (wt/wt). The volumetric and specific productivities were 0.06 g_{PHA}/L.h and 0.02 g_{PHA}/g_X.h, respectively (Table 4.6). The consumed margarine waste was 16.8 g/L, corresponding to a global substrate uptake rate of 0.70 g_s/L.h (Table 4.6). The growth yield was 0.16 g_x/g_s, while the storage yield was only 0.08 g_{PHA}/g_s (Table 4.6).

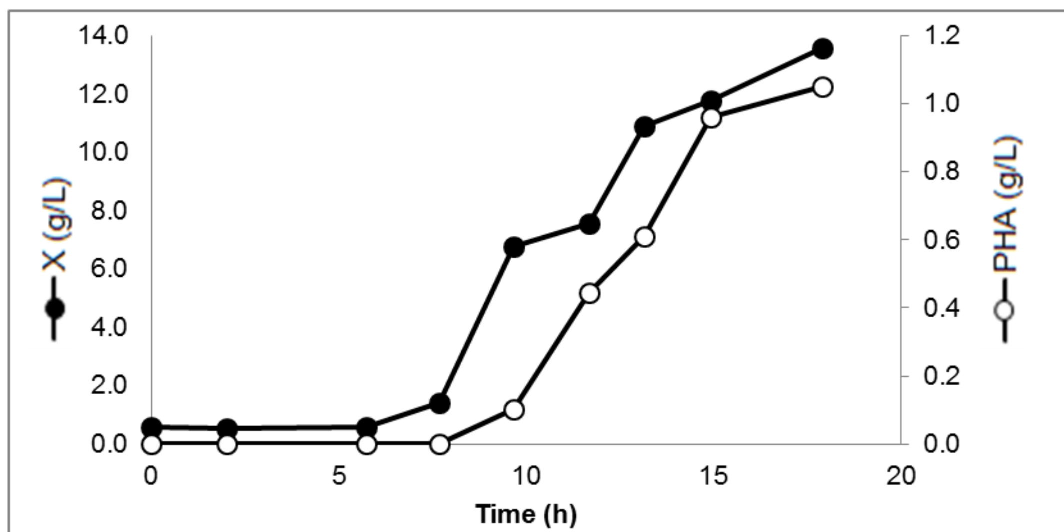

Figure 4.7 – Batch cultivation profile by *C. testosteroni* NRRL B-2611 using margarine waste as carbon source.

The intracellular accumulation of PHA was confirmed by visualization of the Nile Blue stained cells under the optical microscope (Figure 4.8). The epifluorescence was also observed in *C. testosteroni* cells, but it was lower compared to *C. necator* cells due to the low polymer content (7.2%, wt/wt)(Figure 4.8a and 4.8b).

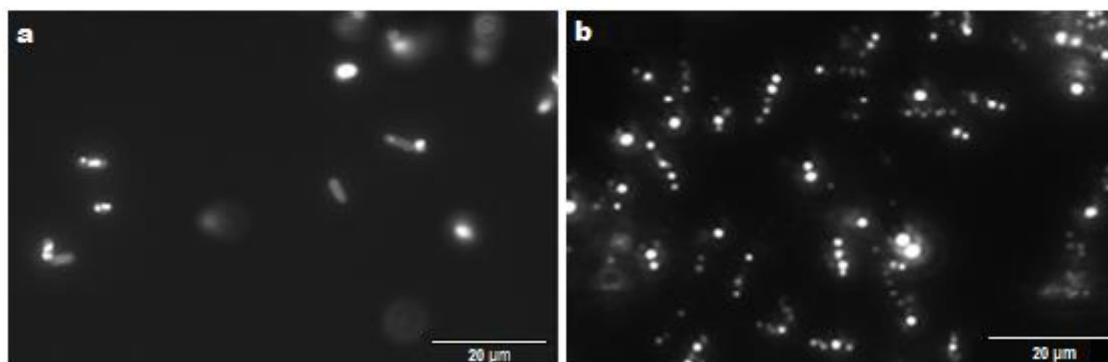

Figure 4.8 – Optical microscopic photographs (100x) of bacterial *C. testosteroni* NRRL B-2611 cells stained with Nile Blue at the beginning (a) and at the end (b) of the batch bioreactor experiment.

Comparing to the shake flask experiment, wherein *C. testosteroni* reached an active biomass concentration of 1.8 g/L (Table 4.5), in the bioreactor batch cultivation, the culture attained a considerably higher cell density (13 g/L) (Table 4.6). But the biomass polymer content was much lower (7.2 and 29.6%, wt/wt, in the bioreactor and shake flask experiments, respectively) (Tables 4.6 and 4.5). Probably, the controlled conditions in bioreactor promoted cellular growth over polymer synthesis. The polymer produced in either experiment was an HB homopolymer (Tables 4.8 and 4.5).

Considerably higher polymer content (78.6–87.5%, wt/wt) has been reported by Thakor et al. (2005) for *C. testosteroni* cultivation on vegetable oils. Further studies on batch bioreactor cultivation with production of copolymers (predominantly HB units) were described by Caballerot

et al. (1995). They used caproate as carbon source and reached a production of 0.72 g/L of CDW, containing 13.9% (wt/wt) of polymer within 74 h. This low cell growth was probably related to the low substrate concentration given to the culture, only 2.2 g/L.

In the present study, the higher biomass concentration and the lower P(3HB) content suggest that, under the conditions used in this study, *C. testosteroni* metabolism was directed towards cell growth instead of accumulation on cultivation with margarine waste. This fact explains the low volumetric productivities and storage yield values comparing with literature ones.

4.3.3 – Batch cultivation experiments with *P. citronellolis* NRRL B-2504

The cultivation profile of *P. citronellolis* cultivated in batch bioreactor with margarine waste as substrate is shown in Figure 4.9. After initial short lag phase of 2 h, the culture grew at a maximum specific growth rate of 0.39 h^{-1} (Figure 7.3, in section 7.2) (Table 4.6). After 7 h of cultivation, the cell growth decreased significantly, while it was observed an increase of the polymer concentration. After 16 h, the CDW was 8.1 g/L with 7.0% (wt/wt) polymer content. At this point 0.6 g/L of polymer concentration and 8.0 g/L of active biomass concentration was achieved. *P. citronellolis* consumed 14.2 g/L of the initial substrate, which corresponds to a growth yield of 0.48 g_x/g_s, a storage yield of 0.04 g_{PHA}/g_s and a global substrate uptake rate of 0.84 g_s/L.h. The PHA volumetric and specific productivities were 0.04 g_{PHA}/L.h and 0.004 g_{PHA}/g_X.h (Table 4.6).

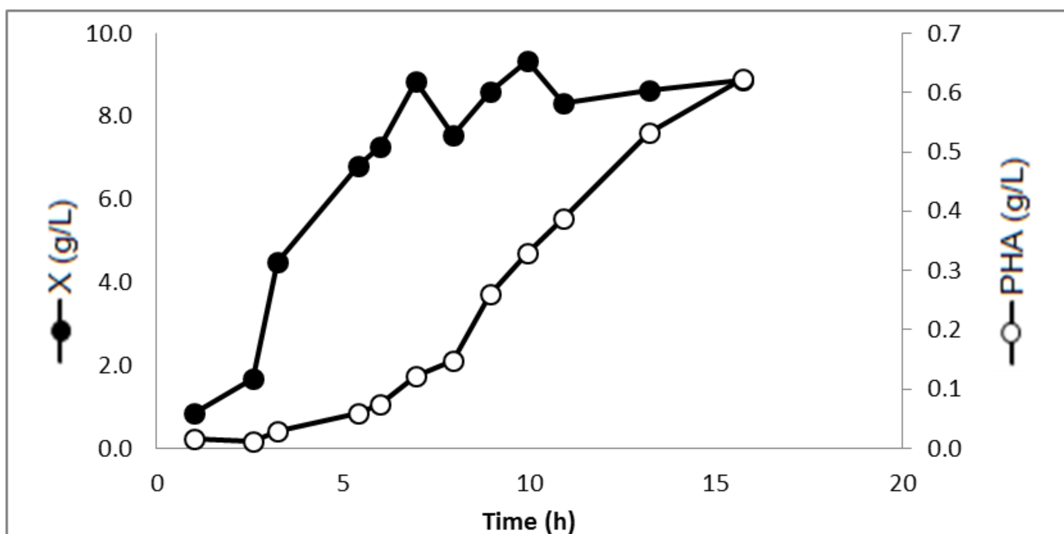

Figure 4.9 – Batch cultivation profile by *P. citronellolis* NRRL B-2504 using margarine waste as carbon source.

P. citronellolis cells stained with Nile Blue were visualized under the optical microscope (Figure 4.10) and polymer granules were visible. However, the epifluorescence was lower compared to *C. necator* cells because polymer content was much lower (7.0%, wt/wt) (Figure 4.10a and 4.10b).

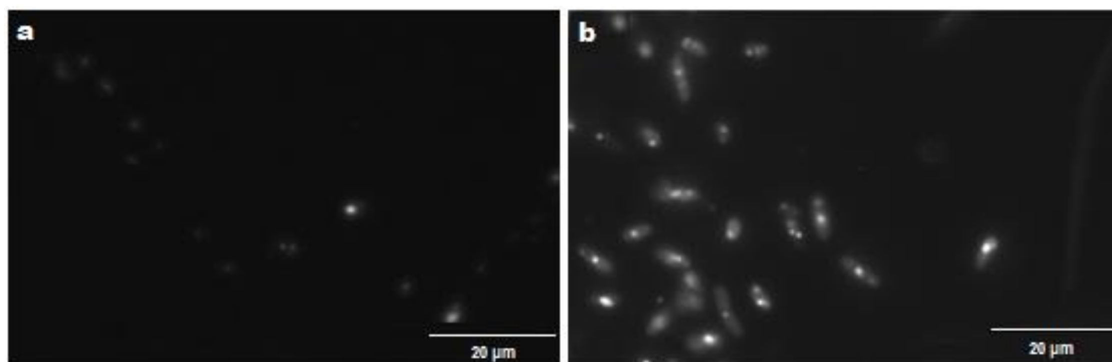

Figure 4.10 – Optical microscopic photographs (100x) of bacterial *P. citronellolis* NRRL B-2504 cells stained with Nile Blue at the beginning (a) and at the end (b) of the batch bioreactor experiment.

PHA content in the biomass was similar (7.0%, wt/wt) (Table 4.6) to the value obtained in the shake flask experiments (8.2%, wt/wt) (Table 4.5). On the other hand, the active biomass concentration has increased from 6.3 g/L to 8.0 g/L in bioreactor experiment. In both experiments, mcl-PHA were produced. However, while the polymer produced in the shake flask assays contained HHx, HO and HD monomers (Table 4.5), in the bioreactor experiment only HO (50.8 mol%) and HD (49.2 mol%) monomers were observed (Table 4.8). This fact could be explained by the substrate complexity or the incomplete degradation of long-chain fatty acids responsible for the incorporation of HHx monomer.

In Muhr et al. (2013) study, *P. citronellolis* produced mcl-PHA from tallow-based biodiesel using a discontinuous fed-batch mode. The $\mu_{máx}$ was 0.10 h^{-1} (lower than that obtained with the margarine waste) and the CDW was 30.6 g/L, after 72 h. Furthermore, a final mcl-PHA content of 27 % (wt/wt) and a volumetric productivity of 0.11 g_{PHA}/L.h were obtained. Despite the low mcl-PHA content obtained with margarine waste, higher substrate concentration (64.7 g/L) was used by Muhr et al. (2013). It should be taken in account that in this study, the experiment was performed in a batch mode, while Muhr et al. (2013) experiment was performed in a discontinuous fed-batch mode. This fact might explain the differences on volumetric productivity values. Also, in contrast with this experiment, Muhr et al. (2013) controlled the pH with NH_4OH solution (25%). This strategy may be used to improve cellular growth of *P. citronellolis* on margarine waste. Although the carbon sources in this study and in Muhr et al. (2013) study were mainly fatty acids, a different composition of mcl-PHA was obtained with HHx, 3-hydroxyheptanoate (HHp), HO, 3-hydroxynonenoate (HN), HD and HHD monomers. These variations in the composition could be related to the substrates composition in fatty acids, as described in section 4.2.

4.3.4 – Batch cultivation experiments with *P. oleovorans* NRRL B-14683

Figure 4.11 shows the cultivation profile for batch bioreactor experiment with *P. oleovorans* NRRL B-14683. The lag phase was only 1 h. The maximum specific growth rate was

0.37 h^{-1} (Figure 7.4, in section 7.2) (Table 4.6) and a maximum CDW of 3.4 g/L was obtained within 20 h. *P. oleovorans* produced PHA, up to a maximum of 8.5% (wt/wt) cell content (Table 4.6). The active biomass and polymer concentration reached 3.4 g/L and 0.3 g/L at the end of the assay, respectively. Thus, it seems that substrate was consumed preferentially for cell growth. This result is confirmed by comparing growth (0.21 g_x/g_s) and storage yields (0.02 g_{PHA}/g_s). The global substrate uptake rate was 0.70 g_s/L.h. The volumetric and specific productivities of polymer were 0.02 g_{PHA}/L.h and 0.004 g_{PHA}/g_X.h, respectively (Table 4.6).

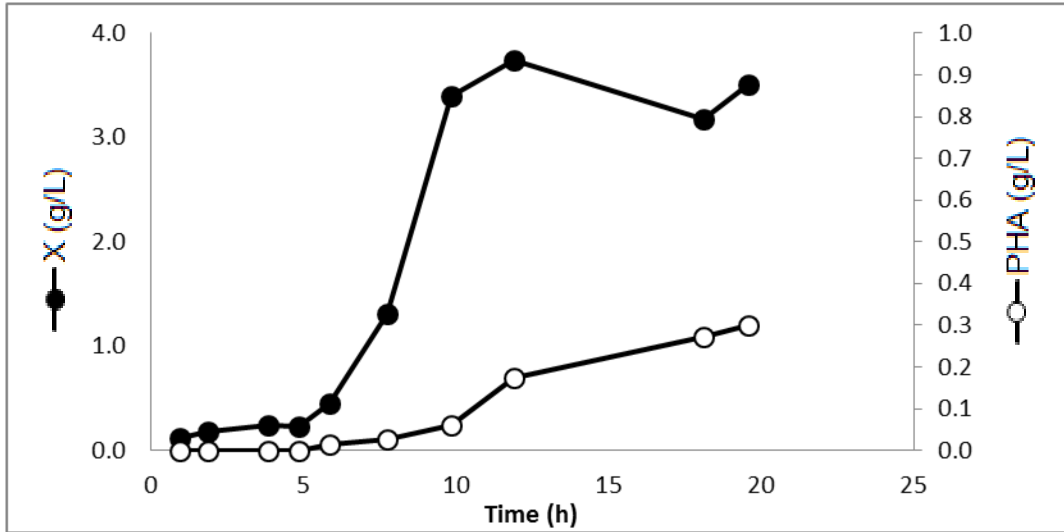

Figure 4.11 – Batch cultivation profile by *P. oleovorans* NRRL B-14683 using margarine waste as carbon source.

Figure 4.12 shows the microscope photographs of *P. oleovorans* NRRL B-14683 cells after staining with Nile Blue. In this case, the morphology of the cells basically did not change, which is in accordance with the lower polymer accumulation (8.5%, wt/wt).

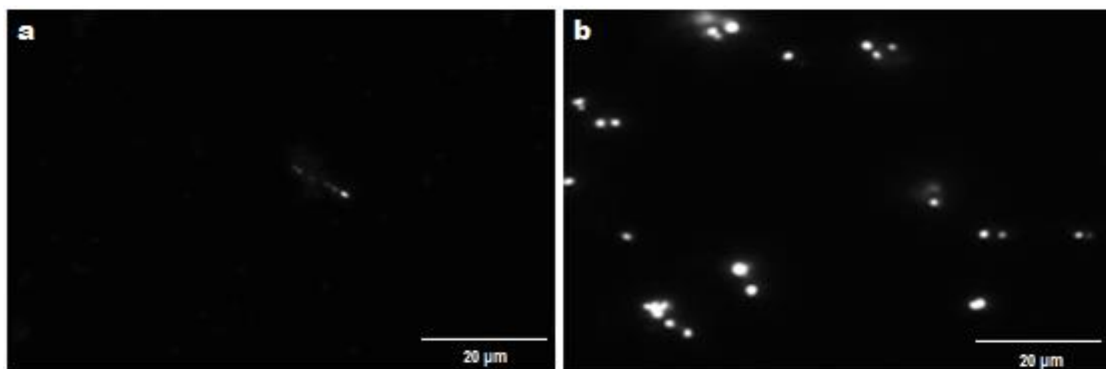

Figure 4.12 – Optical microscopic photographs (100x) of bacterial *P. oleovorans* NRRL B-14683 cells stained with Nile Blue at the beginning (a) and at the end (b) of the batch bioreactor experiment.

The controlled conditions in the bioreactor have contributed to improve PHA production. *P. oleovorans* showed better growth (3.4 g/L of active biomass) and accumulation (8.5%, wt/wt) (Table 4.6) in the bioreactor experiments than in shake flask experiment (2.1 g/L of active

biomass and 1.4% (wt/wt) PHA content) (Table 4.5). In both experiments, the polymer was composed of 70 mol% HO and 30 mol% HD monomers (Tables 4.5 and 4.8).

Compared with PHA synthesis from methylated *Jatropha curcas* seed oil under batch fermentation, *P. oleovorans* reached a lower PHA content (26.1%, wt/wt) (Allen et al., 2010). However, the organism produced the copolymer poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate, P(3HB-*co*-3HV), instead of the mcl-PHA obtained from the margarine waste. On the other hand, mcl-PHA accumulation was reported during growth of *P. oleovorans* in batch culture with octanoate and nonanoate as carbon sources (Durner et al., 2001). In that study, higher maximum specific growth rates (0.48 – 0.59 h^{-1}) were observed. On the other hand, the polymer composition was different: 12 mol% HHx and 88 mol% of HO for growth on octanoate, and 40 mol% HHp and 60 mol% HN for growth on nonanoate. Kim (2002) reported higher mcl-PHA content (75%, wt/wt), as well as higher cell and polymer concentrations (55 g/L and 41 g/L, respectively) when *P. oleovorans* NRRL B-14683 was cultivated in octanoic acid and ammonium nitrate by a fed-batch culture technique. A polymer productivity of 0.6 g_{PHA}/L.h was achieved.

The results of the batch bioreactor cultivation represented an improvement of PHA production over the previous experiments in shake flask, except for *C. testosteroni*. All bacteria were able to use margarine waste for both cell growth and PHA synthesis. When compared with other substrates, like vegetable oils, this carbon source might be a good alternative to produce PHA, mainly using *C. necator*. This organism had the highest cell mass and PHA production and, for this reason, was selected to optimize the cultivation using fed-batch strategies.

4.4 – Fed-Batch experiments

As an attempt to improve *C. necator* performance for reaching high cell concentration and PHA accumulation, fed-batch bioreactor cultivations were performed. Two fed-batch strategies with different types of substrate and nitrogen feeding were implemented. In both strategies, the initial substrate concentration was 20 g/L. Figures 4.13 – 4.14 and Table 4.7 show the concentration profiles for active biomass, polymer concentration and substrate, and the kinetic parameters of each fed-batch experiment, respectively. Also, Table 4.7 presents literature values for the same strain, for comparison. The residual substrate concentration was analysed with the optimized method developed described in section 4.3.

Table 4.7 – Comparison of kinetic parameters for cultivation of *C. necator* DSM 428 in fed-batch bioreactor experiments using margarine waste as carbon source and using soybean oil, n.a.: data not available.

Substrate	$\mu_{máx}$ (h^{-1})	CDW (g/L)	X (g/L)	PHA content (%, wt/wt)	PHA (g/L)	r_{PHA} (g_{PHA}/L.h)	q_{PHA} (g_{PHA}/g_x.h)	r_s (g_s/L.h)	$Y_{x/s}$ (g_x/g_s)	$Y_{PHA/s}$ (g_{PHA}/g_s)	References
Margarine waste	0.14	27.9	6.1	76.8	21.8	0.62	0.101	1.56	0.11	0.40	This work
	0.17	57.5	48.4	15.8	9.1	0.22	0.004	2.44	0.48	0.09	This work
Soybean oil	n.a.	126	30.2	76	95.8	1.00	n.a.	n.a.	n.a.	0.76	Kahar et al. (2004)

4.4.1 Fed-Batch bioreactor experiment with pulse feeding based on DO

In the first fed-batch cultivation (Figure 4.13), the margarine waste feeding was controlled by DO and stirring speed variation. Aiming to attain high cell concentration, the margarine waste was added to the culture after an increase of DO, followed by a reduction of the stirring speed was observed, which were indicative that the metabolic activity of the cells had decreased, probably due to low available carbon source concentration. Hence, fresh substrate was added at that time. Also, during the first 21 h of cultivation, NH_4OH was used for pH control, serving also as the nitrogen source for cell growth, while afterwards it was replaced by NaOH. This was intended to result as a nitrogen limitation for improvment of PHA accumulation at the later stage of the experiment.

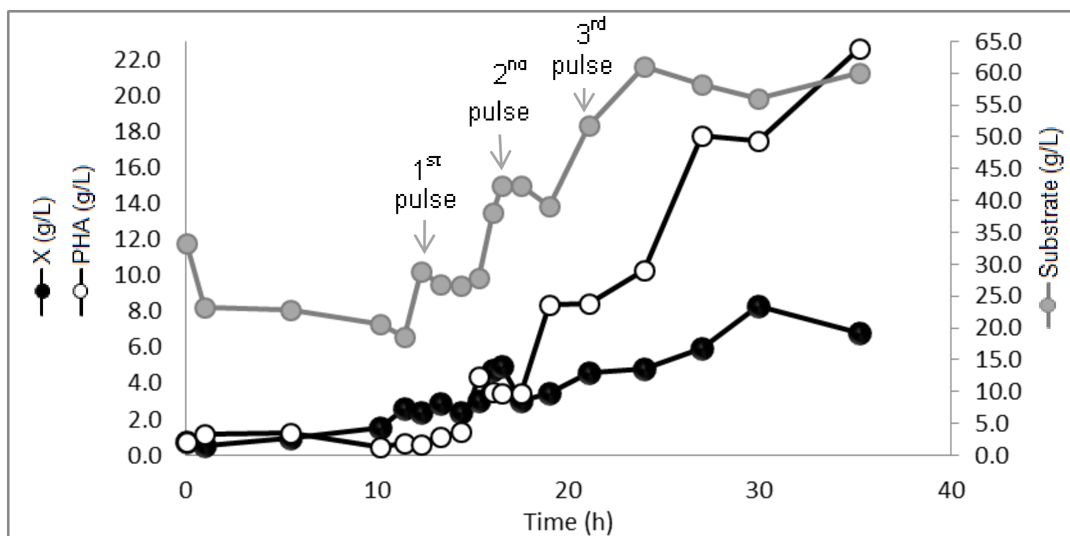

Figure 4.13 – Fed-batch bioreactor profile for cultivation of *C. necator* using margarine waste as carbon source. Concentration of active biomass (X), polymer (PHA) and substrate are shown. The substrate pulse occurred at 12, 16 and 20 h and the pH control was change by NaOH at 21 h.

After a lag phase of less than 5 h, the cells entered the exponential growth phase, achieving a maximum specific growth rate of 0.14 h^{-1} (Figure 7.5, in section 7.3) (Table 4.7). This value is similar to the one obtained in the batch experiment (0.15 h^{-1}) (Table 4.6). The first carbon source pulse was added after 12 h of cultivation, when a rise on the DO (>31%), followed by a significant reduction of the stirring speed, was observed. At that point, the active biomass concentration was only 1.9 g/L, which was lower than that obtained in batch culture at 20 h (4.7 g/L) (Table 4.7 and 4.6, respectively). Two more pulses were added at 16 h and 21 h, based on DO rise and stirring speed drop (Figure 4.13). It was expected that after the addition of the carbon source pulses, the DO would decrease and the stirring speed would increase. However, this behaviour was observed for a very short period of time (a few minutes) and, then, the DO increased again. The active biomass concentration increased to 4.6 g/L before pH control shift from NH_4OH to NaOH. The cells continued to grow up 30 h (Figure 4.13), indicating that there was enough nitrogen available for growth during that period of time. A maximum of 8.3 g/L of active biomass concentration was obtained at 30 h of cultivation.

Looking for polymer concentration, only 0.7 g/L were obtained before substrate pulses. After each addition of margarine waste, this value increased significantly, as shown in Figure 4.13. As expected, when NaOH was used for pH control, the polymer concentration increased substantially, achieving a maximum polymer concentration of 21.8 g/L at the end of the experiment (Table 4.7).

After 35 h, 76.8% (wt/wt) of P(3HB) content, 27.9 g/L of CDW and 6.1 g/L of maximum active biomass concentration were achieved (Table 4.7). The volumetric and specific productivities were 0.62 g_{PHA}/L.h and 0.101 g_{PHA}/g_X.h, respectively. The storage yield was 0.40 g_{PHA}/g_s, growth yield was 0.11 g_x/g_s and the global substrate uptake rate was 1.56 g_s/L.h (Table 4.7).

At the end of the cultivation, the results obtained in this experiment showed that this strategy enhanced both cellular growth and polymer accumulation compared to batch culture (see section 4.3.1), since more carbon source was supplied to the microorganism. Higher values of active biomass and PHB were obtained and the volumetric and specific productivities were also enhanced. Nevertheless, the storage and growth yields were lower (0.40 g_{PHA}/g_s and 0.11 g_x/g_s, respectively) than the yields obtained in the previous experiment (0.50 g_{PHA}/g_s and 0.37 g_x/g_s, respectively) (Table 4.7 and 4.6, respectively). As observed in the batch culture, a large amount of substrate was not consumed by the culture before the first pulse (Figure 13). The inhibition by certain fatty acids, the inability to consume some substrate compounds and the PHA content of cells at beginning of cultivation can explain this fact, as described in section 4.3.1. In this case, the inocula cells contained 51.8% (wt/wt) of PHA and it seems that cells consumed firstly the polymer and then the substrate. Therefore, substrate was not depleted when a new pulse of margarine waste was added, resulting in a gradual substrate accumulation along operation time. This means that larger amounts of substrate were not consumed compared to the batch culture and also the culture might have a long period of starvation between pulses, because it only could consume a part of the available substrate.

On the other hand, a higher global substrate uptake rate was obtained (1.6 g_s/L.h) and higher growth and storage yields would be expected. This result was probably due to the fact that *C. necator* diverted the substrate to maintenance or production of other secondary metabolites (Doran, 1995).

Despite P(3HB) content was similar to that obtained in the literature (76%, wt/wt) (Kahar et al., 2004; Ng et al., 2010), the overall productivity was lower than reported literature values for other lipid-rich substrates. Kahar et al. (2004) achieved higher active biomass and polymer concentration (30.2 g/l and 95.8 g/L, respectively) using soybean oil (Table 4.7). They also reported a high yield of 0.76 g_{PHA}/g_s and volumetric productivity of 1.0 g_{PHA}/L.h (Table 4.7). Besides the use of a different substrate (soybean oil), the different values could be a result of the feeding strategy used in that work (addition of soybean oil, ammonium chloride and magnesium chloride at predetermined time intervals).

As expected, nitrogen limiting conditions, directed *C. necator* metabolic carbon flux towards P(3HB) synthesis, leading to high values of P(3HB) content (Ng et al., 2010). To further improve the bioprocess, it was needed to take into account the feed and pH control strategy and also time of the inocula preparation.

4.4.2 – Fed-Batch bioreactor experiment with periodic pulse feeding

The second fed-batch strategy was based on supplying the culture with substrate pulses at a predetermined time (2 h intervals) (Figure 4.14). NH_4OH was used for pH control throughout the entire experiment.

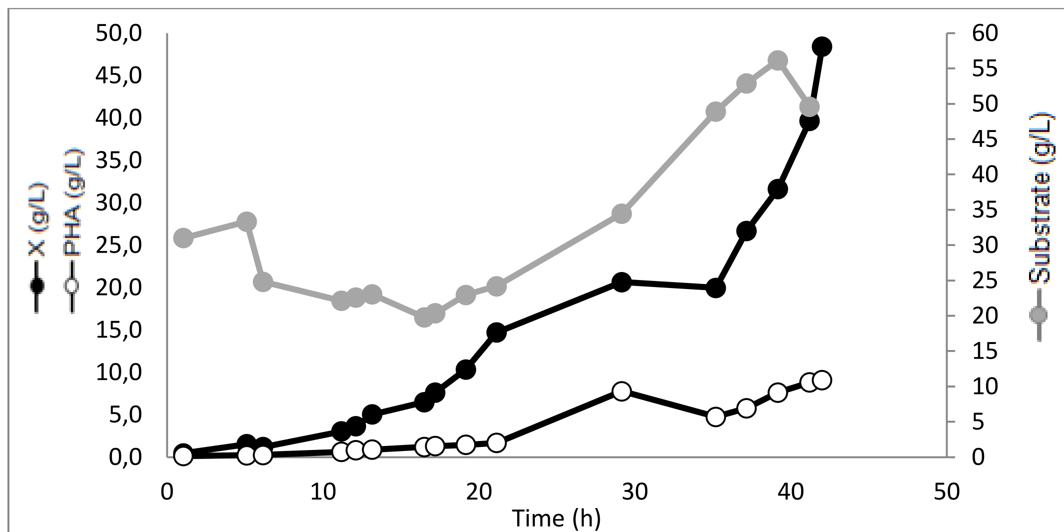

Figure 4.14 – Fed-batch bioreactor profile for cultivation of *C. necator* using margarine waste as carbon source. Concentration of active biomass (X), polymer (PHA) and substrate are shown. The substrate pulses was manual added every 2 h, after 19 h. The pH was control by NH_4OH throughout the entire experiment.

A lag phase similar to the previous fed-batch experiment was observed (less than 5 h). The cells achieved a maximum specific growth rate of 0.17 h^{-1} (Figure 7.6, in section 7.3)(Table 4.7), which was higher than in batch culture (0.15 h^{-1}) and the firs fed-batch (0.14 h^{-1}) (Table 4.6 and 4.7, respectively). At this point, the decrease of the margarine waste concentration was associated to the increase of cells (from an initial active biomass concentration of 1.5 to 10.3 g/L, within 19 h) (Figure 4.14). After this time, the additional amount of margarine waste was intermittently added to culture broth and the active biomass concentration increased significantly, as shown in Figure 4.14.

At the end of this experiment (42 h), 15.8% (wt/wt) of P(3HB) content and 57.5 g/L of CDW were obtained, corresponding to 9.1 g/L of polymer concentration and 48.4 g/L of active biomass concentration (Table 4.7). The volumetric productivity was 0.22 g_{PHA}/L.h and the specific productivity was 0.004 g_{PHA}/g_{X}.h. The storage and growth yields were 0.09 g_{PHA}/g_{s} and 0.48 g_{x}/g_{s}, respectively, and the global substrate uptake rate was 2.44 g_{s}/L.h (Table 4.7).

In this second fed-batch culture, the cellular growth was the highest of all experiments performed in this study with margarine waste (Table 4.7): CDW (57.5 g/L) and active biomass concentration (48.4 g/L). This high cell density was a result of the substrate feeding strategy, but also the maintenance of NH_4OH as pH control which ensured enough nitrogen availability for cell growth throughout the assay. The total amount of substrate added was similar for both fed-batch experiments (between 443 – 456g). But in the second experiment, adding lower amounts of substrate within shorter periods of time might have allowed for the culture to have a better assimilation of margarine waste. Besides that, since the time of inocula was lower (24 h) than in previous experiments (72 h), which might have contributed to the higher maximum specific growth rate and higher active biomass concentration observed. However, a significantly lower P(3HB) content was obtained (15.8%, wt/wt) when compared with the batch (55.8, wt/wt) (Table 4.6) and the first fed-batch (76.8, wt/wt) (Table 4.7) experiments, probably because no nitrogen limitation was implemented. The strategy applied was not effective to achieve high polymer accumulation, as shown by the low values of volumetric and specific productivities (0.22 g_{PHA}/L.h and 0.004 g_{PHA}/g_{X}.h, respectively). Nevertheless, the results are in accordance with the yields obtained, because the growth yield was higher (0.48 g_{x}/g_{s}) than the storage yield (0.09 g_{PHA}/g_{s}). Along the operation time, the substrate was never depleted after a new pulse (Figure 4.14). The margarine waste accumulation was observed, but after 39 h of cultivation, the substrate consumption increased significantly, corresponding to the high increased of active biomass concentration. Thus, the growth yield was slightly higher than obtained in batch culture (0.37 g_{x}/g_{s}). In fact, the growth yield still lower, because culture had not capacity to consume all the components of the margarine waste, as described in section 4.3.1.

Since a large amount of substrate was available and cellular growth was favoured, the global substrate uptake rate was higher (2.44 g_{s}/L.h) than in previous experiments (0.51 and 1.56 g/L.h for batch and first fed-batch, respectively). With this higher value it would be expected again better yields than in batch culture which can be explain by cellular maintenance or secondary metabolites (see section 4.4.1).

This fed-batch experiment demonstrated an efficient cellular growth compared with Kahar and co-workers (2004) study (30.2 g/L of active biomass concentration) (Table 4.7). However, these differences are related with metabolism of bacteria. Regarding to Kahar and co-workers (2004), when phosphorus limitation was applied, the substrate was mainly diverted for PHA production since they obtained a storage yield of 0.76 g_{PHA}/g_S (Table 4.7).

These results showed that this strategy was adequate to improve cells growth but not to accumulate polymer. Further improvement of this process should involve the limitation of an essential nutrient, such as nitrogen, that could increase PHA accumulation by *C. necator*.

4.5 – Thermal characterization of polymer

The thermal properties of the polymers obtained in all bioreactor experiments with each bacterial culture have been determined by Differential Scanning Calorimetry (DSC). The results are shown in Table 4.8. Due to the low amount of polymer extracted from cultivation with *P.oleovorans* NRRL B-14683, it was not possible to analyse this polymer.

Table 4.8 – Thermal properties for the polymers produced by the different bacteria determined by DSC analysis, n.a.: data not available.

Bacteria	Mode	Polymer	T_g (ºC)	T_m (ºC)	ΔH (J/g)	χ_c(%)	Reference
C. necator **DSM 428**	Batch	P(3HB)	7.9	173.4	82.6	56.6	This work
	Fed-batch	P(3HB)	1.1	169.3	71.1	48.7	This work
	Fed-batch	P(3HB)	0.5	169.9	73.1	50.1	This work
C. testosteroni **NRRL B-2611**	Batch	P(3HB)	0.6	160.2	17.2	11.8	This work
A. caviae	Batch	P(3HB)	4.0	177.0	97	n.a.	Doi et al. (1995)
C. necator **DSM 428**	Three stages	P(3HB)	n.a.	170.0	84.7	n.a.	López-Cuellar et al. (2011)
P. citronellolis **NRRL B-2504**	Batch	mcl-PHA (HO,HD)	-45.6	60.8	1.0	0.7	This work
P. citronellolis **NRRL B-2504**	Fed-batch	mcl-PHA (HHx, HHp, HO, HN, HD, HHD)	-43– -48	48–54	20	10-12	Muhr et al. (2013)

The P(3HB) polymers produced by *C. necator* DSM 428 had a T_m in the range 169.3–173.4ºC, with ΔH of about 73.1–82.6 J/g (Table 4.8). These values can be regarded as values typical for this type of PHA. Doi and co-workers (1995) reported a T_m of 177ºC, and ΔH of 97 J/g, while López-Cuellar and co-workers (2011) reported a T_m of 170ºC with ΔH about of 84.7 J/g. Rather differing T_g values were obtained for the polymers produced in the different experiments (Table 4.8). The polymer produced in the batch experiment had a higher T_g (7.9ºC)

compared to that obtained in the fed-batch experiments (0.5–1.1ºC). Nevertheless, the values obtained in this study are in the range of the values reported in the literature (-4 to 9ºC) for P(3HB) produced by *C. necator* (Doi et al., 1995; Laycock et al., 2012) . Based on the melting enthalpies, the crystallinity of P(3HB) was in the range of 48.7–56.6%. The relatively high crystallinity indicated that the polymer was a rigid and brittle material with poor impact strength, limiting its used in some final applications that need a high impact resistance (Laycock et al., 2012).

C. testosteroni NRRL B-2611 also produced a P(3HB) with similar T_g (0.6ºC) and Tm (160.2ºC) values, but the ΔH decreased to 17.2 J/g, which led to a lower crystallinity (11.8%). Differences in crystallization temperature or time (at a specific temperature) and annealing treatment between samples can affect properties as well as processing conditions, which can explain the different values for P(3HB) produced by *C. testosteroni* NRRL B-2611 (Laycock et al., 2012).

As expected, the thermal properties of the mcl-PHA produced by *P. citronellolis* NRRL B-2504 were significantly different. Lower T_g and T_m values, -45.6 and 60.9ºC, respectively, were obtained (Table 4.8). The polymer also showed a ΔH of 1 J/g, with a crystallinity of 0.7% (Table 4.8). Contrary to P(3HB), mcl-PHA have decreased brittleness and stiffness, and increased flexibility, which makes this polymer practically an amorphous material (Laycock et al., 2012). Similar values were reported for mcl-PHA produced by *P. citronellolis*, namely, T_g varying in the range of -43 to -48ºC and T_m of 48 to 54ºC (Muhr et al., 2013). But the crystallinity was in the range of 10–12% due the higer value of ΔH (about 20 J/g). This might be due to the effect of microstructure and, in particular, the influence of compositional distribution/blending on mechanical properties. In this study, only HO and HD monomers were detected in the mcl-PHA produced by *P. citronellolis*, while Muhr and co-workers (2013) achieved HHx, HHp, HO, HN, HD and HHD monomers (Table 4.8). Also the different monomers composition can contribute to a different crystallization behaviour (Laycock et al., 2012).

5 Conclusion and outlook

One of the reasons why PHAs are not yet competitive against petrochemical-based common polymers is the relatively high production costs of these biopolymers. As a practical application, the use of renewable feedstock can be considered as a cost-effective way for PHA production. This current research investigated the potential of fat wastes from margarine production to produce PHA.

The three fat wastes (margarine and refinery wastes and sludge) presented different physical and chemical properties. Margarine and refinery wastes are rich sources of FFAs (56-76%) with a high fatty acid content. These fat wastes possessed a low pH (1.75–4.03) and water contents (1.11–8.90), a density of about 0.9 g/cm^3, as well as the great solubility in hexane (values). In contrast, the sludge arising from the mixture of several effluents from the manufacturing plant, showed a high water content (77.92–87.55) and consequently lower fatty acids content, density and solubility. This waste was characterized by a high heterogeneity. However, a similar fatty acid profile was determined for all fat wastes, containing high percentage of oleic (37–54%), linoleic (4–27%), stearic (11–18%) and mystiric (4–14%) acids and small amounts of other acids (palmitic and linolenic acid).

It was shown that the fat wastes can be used to synthesize both P(3HB) and mcl-PHA depending on the bacterial strain used. The shake flask experiments demonstrated that *C. necator* DSM 428 was able to assimilate all three fat wastes and convert them to P(3HB), but the margarine waste presented the best results in terms of polymer production (69.4% (wt/wt) P(3HB) content in the biomass). Several bacterial strains have been screened in shake flask experiments and *C. necator* DSM 428, *C. testosteroni* NRRL B-2611, *P. citronellolis* NRRL B-2504 and *P. oleovorans* NRRL B-14683 showed the best polymer producing performance using margarine waste. The first two bacteria produced P(3HB), while both *Pseudomonas* species were able to produced mcl-PHAs, consisting primarily of HO and HD monomers. Such strains were selected for batch bioreactor cultivation experiments, in which the highest PHA cell content was obtained for *C. necator* DSM 428 (55.8%, wt/wt), achieving a volumetric productivity and storage yield of 0.33 g_{PHA}/L.h and 0.50 g_{PHA}/g_S, respectively. *C. testosteroni* showed a weaker performance compared to *C. necator*, since only 7.2% (wt/wt) PHA content in the biomass was obtained. Regarding the tested *Pseudomonas* species, *P. citronellolis* NRRL B-2504 had the highest active biomass (8 g/L) and polymer production (0.6 g/L).

Two fed-batch feeding strategies were evaluated, in order to improve both cell growth and P(3HB) production by *C. necator* DSM 428, which showed the best performance in the batch experiments. Pulse feeding based on DO increase and stirring rate drop, resulted in higher PHA production (21.8 g/L) due to the higher availability in substrate (margarine waste) supplied to the culture and the nitrogen limiting conditions. On the other hand, the periodic substrate feeding, concomitant with nitrogen availability, resulted in increased biomass production (48.4 g/L).

Considering the thermal characterization, the P(3HB) analyzed in this work presented low T_g and a high melting point and crystallinity (11.8–56.6%), similarly to reported values for P(3HB) polymers. The mcl-PHA produced by *P. citronellolis* NRRL B-2504 and *P. oleovorans* NRRL B-14683, composed of HO and HD monomers, had -45.6ºC of T_g, 60.8ºC of T_m and 0.7% of crystallinity.

Future studies have to be carried out to determine the effects of varying the culture conditions on the rate of accumulation, as well as on the properties of the polyester formed. When compared with other substrates, such as soybean oil or jatropha oil, margarine waste demonstrated to be an interesting alternative as carbon source, because similar PHA content (76%, wt/wt) were obtained from those oils (Kahar et al., 2004; Ng et al., 2010). Nevertheless, the conditions for bacterial cultivation using this substrate still have to be optimized. Future experiments on bioreactor scale have to focus not only the increase of intracellular PHA content but also the high cell density. In this way, the following strategies should be implemented.

As future work, this process can be improved by different strategies, including:

- evaluate the influence of mineral medium composition on the margarine waste carbon source, soapstock formation and its consumption by bacteria, as well as lipid and fatty acid profile over time of cultivation. This knowledge is important to understand why the tested bacteria were not able to consume all the supplied margarine waste as substrate.
- evaluate the ammonium concentration over time to determine the best nitrogen concentration and avoid early cell growth limitation. A different nitrogen source could be implemented such as urea, since it was proved as the most suitable nitrogen source for *C. necator*, increasing significantly both cell biomass and PHA production (Ng et al., 2010).
- implement two different feeding strategies in the one fed-batch cultivation. In the first stage, a margarine waste and NH_4OH feeding strategy should be employed during the active growth phase without any nitrogen limitation, similar to which was used in section 4.4.2. To a high specific growth rate, the concentration of margarine waste in culture broth could remained at around 20 g/l (Kahar et al., 2004). The cell concentration must be high at the beginning of PHA accumulation phase. In a second stage, a continuous carbon source feeding and nitrogen limitation should be introduced to enhance PHA production, as in second phase of section 4.4.1.
- implement a fed-batch cultivation for *C. testosteroni* NRRL B-2611, *P. citronellolis* NRRL B-2504 and *P. oleovorans* NRRL B-14683 with focus on high cell density and high intracellular PHA content.
- implement a continuous culture strategy to achieve high productivity seems to be a good alternative (Atlić et al., 2011). Although the high complexity of the technical set-up and a higher risk for microbial contamination, this strategy could be used for a broad range of potential applications of the products, due to the flexibility of the system by triggering the polymer composition by co-substrate additions in each vessel.
- biopolymer characterization in terms of average molecular weight and polydisperisty.

6 References

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7. Appendix

7.1 Fat wastes characterization

Table 7.1 – Calibration solutions using in lipid profile analysis. Each component concentration is expressed in µg/mL.

Standard solution	Butanetriol	1.3-Dilolein	Glicerol	Mono olein	Tricaprin	Triolein
1	80	50	5	250	800	50
2	80	200	20	600	800	150
3	80	350	35	950	800	300
4	80	500	50	1250	800	400

7.2 – Batch bioreactor cultivation experiments

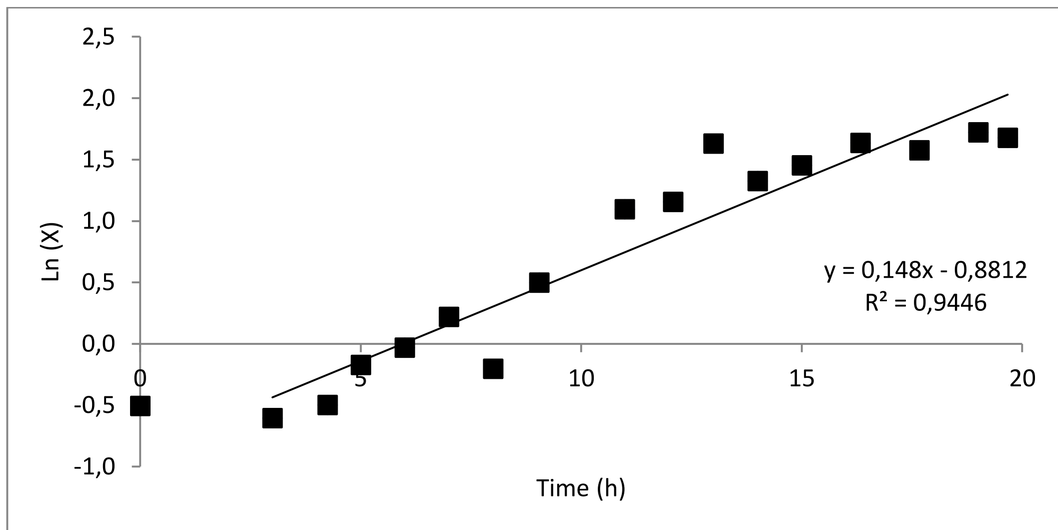

Figure 7.1 – Maximum specific growth rate determination for *C. necator* DSM 428 in batch cultivation using margarine waste as carbon source.

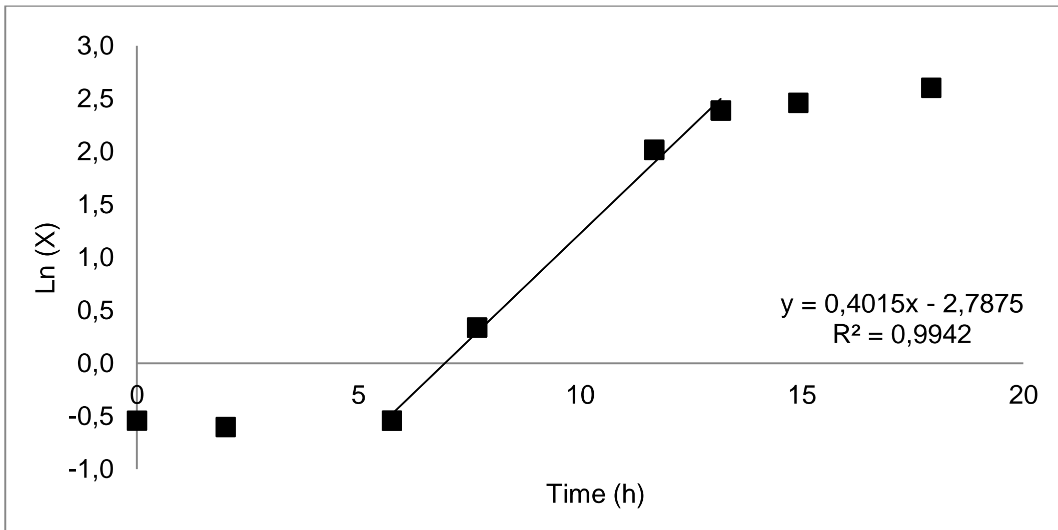

Figure 7.2 – Maximum specific growth rate determination for *C. testosteroni* NRRL B-2611 in batch cultivation using margarine waste as carbon source.

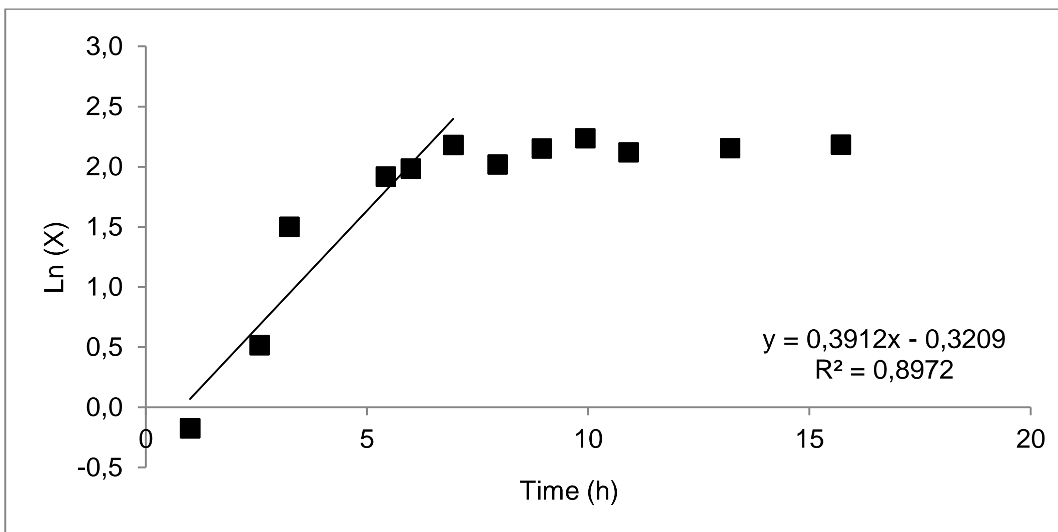

Figure 7.3 – Maximum specific growth rate determination for *P. citronellolis* NRRL B-2504 in batch cultivation using margarine waste as carbon source.

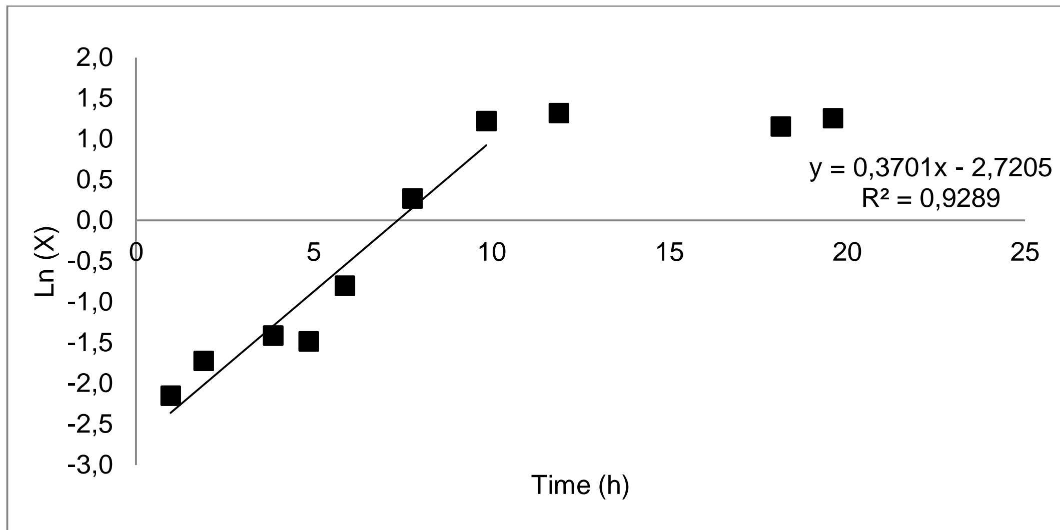

Figure 7.4 – Maximum specific growth rate determination for *P. oleovorans* NRRL B-14683 in batch cultivation using margarine waste as carbon source.

7.3 – Fed-batch bioreactor cultivation experiments

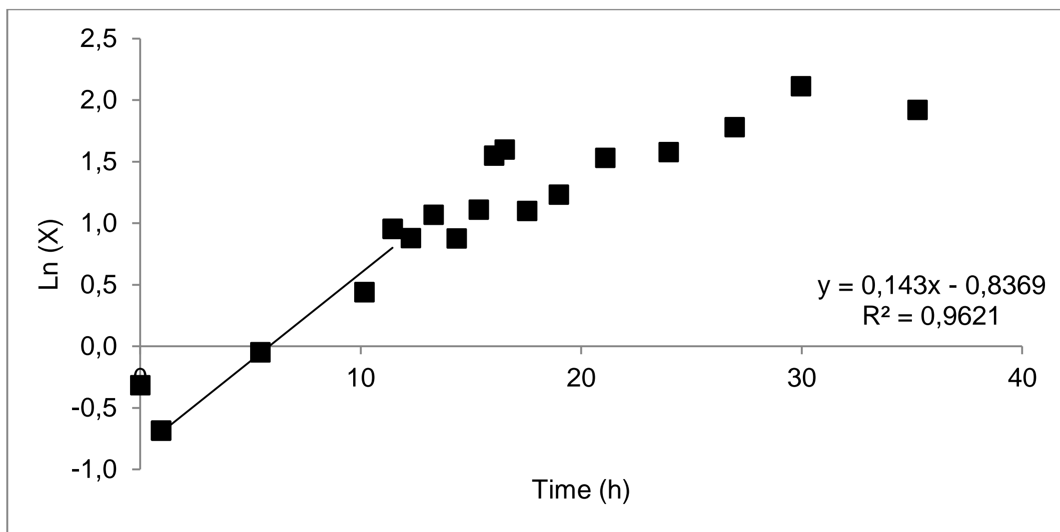

Figure 7.5 – Maximum specific growth rate determination for *C. necator* DSM 428 in first fed-batch cultivation using margarine waste as carbon source.

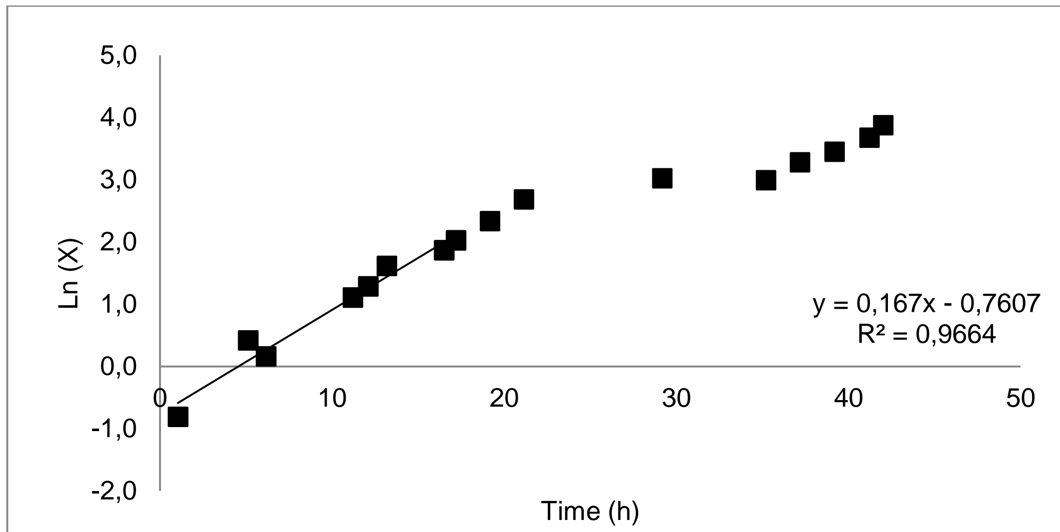

Figure 7.6 – Maximum specific growth rate determination for *C. necator* DSM 428 in second fed-batch cultivation using margarine waste as carbon source.