Tutor/s

Dr. Joan Mata Álvarez
Departament d'Enginyeria Química

Grau en Enginyeria Química

Treball Final de Grau

Bioplastic production from wastes and wastewater
Producció de bioplàstics a partir de residus i aigües residuals

Esther Alcaraz Cercós

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Universitat de Barcelona

Dos campus d'excel·lència internacional
B:KC Barcelona Knowledge Campus
HUB Health Universitat de Barcelona Campus

REPORT

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1. SUMMARY

Dependence on conventional plastics and their boundless usage have resulted in waste accumulation and greenhouse gas emissions. Part of a solution lies in using biodegradable plastics such as polyhydroxyalkanoates (PHA), which can be produced by bacterial fermentation. The interest in these biopolymers lies in its similar properties to conventional plastics. However, its price is still too high to compete in the current market. One way to produce PHA reducing the cost of production is to use wastes and wastewater as a raw material. They have not only a great capacity to produce PHA with a high potential for accumulation but also to reduce the costs of the waste and wastewater treatment. The process to produce PHAs from waste consists of three steps: an anaerobic fermentation process, the enrichment of the culture and the accumulation of PHA. The fermentation process, to produce volatile fatty acids, is required when carbohydrate wastes are used as substrate. The main challenge in PHA mixed culture processes is the enrichment of PHA-accumulating organisms, which can be carried out with transient conditions of carbon supply, *i.e.* feast-famine regime. This process configuration creates periods of excess and lack of external carbon substrate. Following a certain period of time in the absence of external carbon substrate, a decrease of the amount of intracellular components required for cell growth (RNA and enzymes) occurs. Therefore, when carbon substrate becomes available again, the amount of growth enzymes may not be enough to ensure that a maximum growth rate is reached. On the contrary, fewer enzymes are required for PHA storage and, therefore, PHA storage can occur at a much faster rate than cell growth, thus providing the cells with a means of rapidly consuming the available external substrate. This work presents an economic analysis of PHA production by bacterial fermentation from municipal wastes. Based on an annual production of 2503 tones of PHA, and taking into account the performance of production processes of PHA is 0.33 g PHA/ g VFA-COD approximately, the economic potential which is obtained is not still sufficiently positive. However,

if this investigation line continues, it has been seen that the process can be viable and an effective industrial wastewater treatment technology.

Keywords: polyhydroxyalkanoates, PHA, mixed microbial cultured, wastes, bioplastic, VFA.

2. RESUM

La dependència dels plàstics convencionals i el seu ús sense límits han donat lloc a l'acumulació de residus i emissions de gasos de l'efecte hivernacle. Part d'una solució rau en l'ús de plàstics biodegradables com ara els polihidroxialcanoats (PHA), que poden ser produïts mitjançant una fermentació bacteriana. L' interès en aquests biopolimers rau en les seves semblants propietats als plàstics convencionals, tot i això, el seu preu encara és massa alt per competir en el mercat actual. Una manera de produir PHA reduint els costos de producció és utilitzant residus i aigües residuals com a matèria prima, ja que, per una banda tenen una gran capacitat per produir PHA amb un elevat potencial d'acumulació, i per altra banda, s'aconsegueix reduir els costos de tractament de residus i d'aigües residuals. El procés de producció de PHA en cultiu mixt consta principalment de tres etapes: fermentació acidogència, enriquiment del cultiu i acumulació de PHA. El procés de fermentació, per produir àcids grassos, es requereix quan els residus d'hidrats de carboni s'utilitzen com a substrat. El principal repte en la producció de PHA en cultiu mixt és l'enriquiment dels organismes acumuladors de PHA, el qual és dut a terme mitjançant el subministrament de substrat sota condicions de *feast-famine*. Aquesta configuració crea períodes d'excés i d'escassetat de substrat de carboni extern. Després d'un cert període de temps en absència de substrat de carboni extern, es produeix una disminució de la quantitat de components intracel·lulars necessaris per al creixement cel·lular (ARN i enzims). Per tant, quan el substrat de carboni torna a estar disponible, la quantitat d'enzims de creixement pot no ser suficient per assegurar que s'arriba a una taxa de creixement màxim. Per contra, es requereixen menys enzims per a l'emmagatzematge de PHA i, per tant, l'emmagatzematge PHA pot ocórrer a un ritme molt més ràpid que el creixement cel·lular, proporcionant així les cèl·lules amb un mitjà de consumir ràpidament el substrat extern disponible. Aquest treball presenta un anàlisis econòmic de la producció de PHA mitjançant la fermentació bacteriana dels residus municipals. Basada en una producció anual de 2503 tones de PHA, i tenint en compte que el rendiment del procés de producció de PHA és de 0.33 g PHA/g AGV-DQO aproximadament, el potencial econòmic que

s'obté no és suficientment positiu, tot i això, s'ha demostrat que si es continua investigant en aquesta línia, aquest pot ser un procés viable amb una tecnologia de tractament d'aigües residuals industrials efectiva.

Paraules clau: polihidroxialcanoats, PHA, cultiu mix microbià, residus, bioplàstic, AGV.

3. INTRODUCTION

Plastic products possess many desirable properties such as strength, durability, resistance to degradation and low density, but the accumulation of plastic oil is a serious problem for the environment. These products are an essential part of all industries and in many cases have replaced the use of paper and glass as a packaging material. Its annual consumption has increased from 5 million tones in 1950 to over 250 million tones in the current year (González García *et al.* 2013). The main problem is that they are resistant to degradation, so unlimited use of them generates waste causing their accumulation in the environment with an approximate value of 25 million tones per year, of which 60% is deposited marine environments. This fact brings serious problems, among them the death of animals. Researchers have tried to solve the problem of the accumulation through incineration process, recycling or reuse. However, these solutions are not considered 100% as they have disadvantages such as release of hydrocyanic acid and hydrochloric acid in the case of incineration. These acids are highly hazardous to health. Another important issue is the availability and price of plastic raw material: oil. The production of plastics from this nonrenewable source, oil consumption, is increasing quickly and it will have an impact on its price increasing it drastically.

Taking these facts into account, the synthesis and use of degradable plastic instead of plastic oil seems to be the best solution (Albuquerque *et al.* 2008). These plastics are produced by fermentation using several bacteria, and generally called polyhydroxyalkanoates (PHA). The first discovered PHA was the polyhydroxybutyratre (PHB). PHB and PHV (polyhydroxyvalerate) are produced at industrial scale. However, the high cost of producing these bioplastics and the availability of low-cost petrochemical-derived plastics led to bioplastics being ignored for a long time. The concern over petrochemical plastics in the environment has created a renewed interest in biologically derived polymers. During recent years, intensive research has investigated the bacterial production of PHAs and a great effort is underway to improve this procedure. Nonetheless, the PHA production price is still far above the price of conventional plastics (Salehizadeh and Van Loosdrecht *et al*. 2004). In order to make the process

economically attractive, many goals have to be addressed simultaneously. Recombinant microbial strains are being developed to achieve both a high substrate conversion rate and close packing of PHAs granules in the host cell. A more efficient fermentation process, better recovery and purification, and the use of inexpensive substrates can also reduce the production cost.

PHA production from waste streams using microbial enrichment cultures is a promising option for cost reduction of both PHA polymers and treatment of industrial wastewater. Integration of waste-based PHA production into industry would encompass direction of a suitable waste stream towards a PHA production facility, likely in the proximity of the waste source. The organic compounds in the waste stream are converted to sludge with an ideally high PHA content. At the same time a clean effluent is produced for discharge to surface waters in accordance with local legislation. Furthermore, the PHA containing sludge can be processed and purified into marketable PHA polymer product or used as feedstock for other processes (Morgan-Sagastume *et al.* 2015 and Wang *et al.* 2014). High concentrations of fermentable COD, relatively low nitrogen and solid concentrations and low toxicity promote process feasibility, so food and paper industry effluents may be considered the most suitable substrates for waste-based PHA production. Other waste streams that may be interesting for PHA production include leachate from the composting industry and municipal wastewater, but it should be noted that these streams pose additional challenges like the relatively high nitrogen content and the presence of solids, so the cost of the process increase.

Although there are a large number of factors that influence the feasibility of waste-based PHA production, the most expensive part is the downstream process. A part from optimization this stage, the process feasibility can be improved by increasing the PHA content of the biomass and reducing the chemical products used. The cyclic presence and subsequent absence of volatile fatty acids (VFA) provides a competitive advantage for PHA storing species.

4. OBJECTIVES

The objectives of this present work are:

- To do a review literature in order to see what the polyhydroxyalkanoates are and what are their properties and applications.
- To carry out an overall revision about the stages of production of PHAs from waste and wastewater.
- To study the economic potential that has the PHA production process using OFMW as raw material.

5. POLYHYDROXYALKANOATES

5.1. DEFINITION AND STRUCUTURE OF POLYHYDROXYALKANOATES

Polyhydroxyalkanoates or PHAs are linear polyesters that some bacteria accumulate inside as a source of carbon and energy. Among all known classes of bio-based polymers with plastic-like properties, PHAs are the only ones that are entirely produced and degraded by living cells. The general structure and nomenclature of these compounds is shown in Figure 1. The polymerization of hydroalkanoics acids by the action of intracellular enzymes, take place through the condensation the group of carboxyl group monomer (acid hydroxyalkanoic), with the hydroxyl group of the next monomer to form an ester bond (Sudesh *et al.* 2007). The granule of PHA is accumulated as a liquid polymer, mobile and amorphous form of inclusion bodies in the cytoplasm microbial surrounded by a monolayer of phospholipids containing polymerases and despolimerases enzyms. PHA can represent 90% of the cell dry weight as it shown in Figure 2 (Verlinden *et al*. 2007).

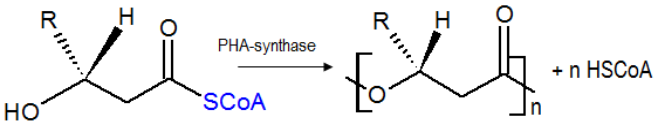

Figure 1. Synthesis of PHA in bacteria using hydorxyacyl-CoA thioesters as precursor.

(image from Verlinden et al. 2007)

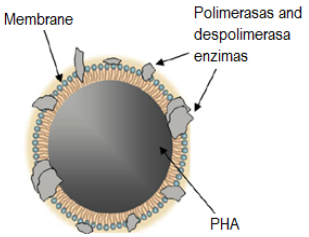

Figure 2. Scheme of PHA granule accumulated intracellular.

(image from Shudesh et al. 2007)

Research on accumulation process of PHA indicate that the number of granules per cell is defined in the first stages of the accumulation and the production of the polymer ceases when its content reaches about 80% of dry cell weight. This fact has led to the conclusion that there are physical constraints that prevent to the cell accumulate more polymer, despite of the availability of substrate and enzyme activity of PHA-polymerase (González García *et al.* 2013).

These inclusions can be observed in a microscopy as spherical granules of different sizes as shown in Figure 3.

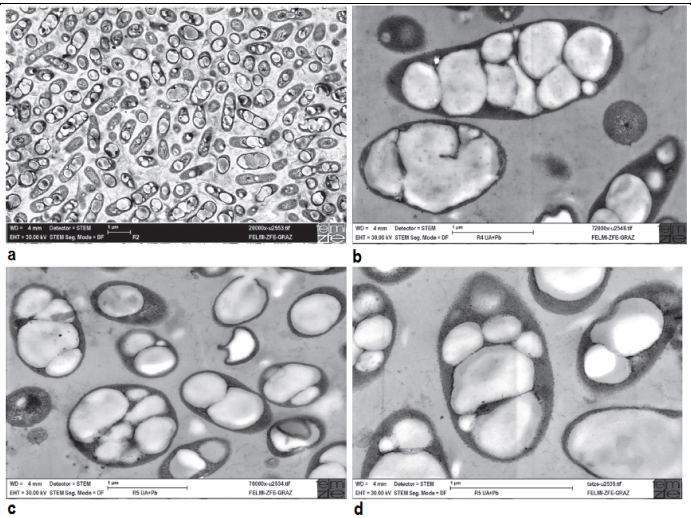

Figure 3. Electron-microscopic pictures of polyhydroxyalkanoate (PHA)-rich Cupriavidus necator DSM 545 cells cultivated in a continuous fermentation process on glucose. Magnification: ×20,000 (**a**), ×72,000 (**b**), ×70,000 (**c**) and ×150,000 (**d**). Percentages of PHA in cell mass: 48% (**a**), 65% (**b**) and 69% (**c** , **d**).
(Image from Elisabeth Ingolić, *FELMI-ZFE-Graz*)

The pendant group (R in Fig.1) varies from methyl (C_1) to tridecyl (C_{13}). In all PHA that have been characterized so far, the hydroxyl-substituted carbon atom is of the stereochemical R-configuration. There is an enormous variation possible in the length and composition of the side chains. This variation makes the PHA polymer family suitable for an array of potential applications.

The structure of PHA composed of 3-hydroxy fatty acids is shown in Figure 4. The most common polymers, with structure given in Figure 4, are shown in Table 1. The value of n in Figure 4 depends on the pendant group and the micro-organisms in which the polymer is produced. The n value is typically between 100 and 30.000 (Andler and Díaz-Barrera *et al*. 2013).

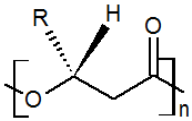

Figure 4. Poly(3-hydroxyalkanoates)

(image from Verlinden et al. 2007)

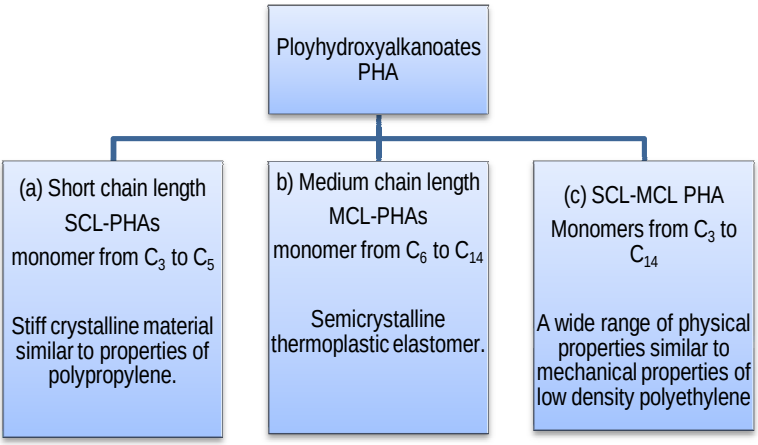

Figure 5. General structure and three types of polyhydroxyalkanoates (PHAs). Their characteristics are also summarized.

Table 1. PHAs and corresponding R groups.

R group	Full name	Short name
CH_3	Poly(3-hydroxybutyrate)	PHB
CH_2CH_3	Poly(3-hydroxyvalerate)	PHV
$CH_2CH_2CH_3$	Poly(3-hydroxyhexanoate)	PHHx

Regarding its monomeric structure, PHA can be classified according to the chain length of the fatty hydroxyalkanoics which are constituted. There are three types of polyhydroxyalkanoates: (a) short chain length hydroxyalkanoic acids (PHA_{SCL}) with an alkyl side chain, which are produced by *Ralstonia eutropha* and many other bacteria. PHA_{SCL} contain 3-5 carbon atoms, for example poly-3-hydroxybutyrate (P3HB), poly-4-hydroxybutyrate (P4HB); (b) medium chain length hydroxyalkanoic acids (PHA_{MCL}) with alkyl side chains that are produced (Chaitanya *et al*. 2014). PHA_{MCL} contain 6-14 carbon atoms and (c) long chain length (PHA_{LCL}) obtained from long chain fatty acids, which contain more than 14 carbon atoms (Fig. 5).

The monomer composition, molecular structure and physical chemical properties of PHAs vary, depending on the producer organism as well as on the carbons source used for de growth. The monomer composition of PHA has considerable effects on its physical properties.

The PHA structure can effectively be controlled by adjusting the carbon substrates to achieve desired monomer contents, by engineering metabolic pathways on the hosts or by feeding the culture with carbon substrates containing functional side chain that in a second step can suffer chemical modifications (González García *et al*. 2013).

5.2. PHYSICAL AND CHEMICAL PROPERTIES

PHAs have similar physical properties to those of petroleum-based plastics, such as polypropylene and polyethylene, but they have the advantage that they can be synthesized from renewable carbon, are biodegradable (can be assimilated by many microorganisms either soil, seas, lakes or sewage) and are biocompatible (not causing toxic effects). These properties give them great importance as substitutes for conventional plastics (Harding *et al*. 2007).

Bacteria produce PHAs with average molecular mass (M_n) of up to 4.0 x 10^6 Da with a polydispersity (M_w/M_n) of around 2.0. In regards to the molecular weight of PHA, it depends on the conditions of production and recovery of these compounds (Verlinden *et al.* 2007). The mechanism that affect and determine the molecular weight of the PHA in bacterial cells are not yet entirely understood, but is generally attributed mainly to the kind of microorganism and the carbon source used. Meanwhile, the recovery method of the polymer can influence the molecular weight significantly. The extraction with organic solvents leads polymers with higher molecular weight, compared to the extraction sodium hypochlorite or other chemicals. Because of this, there are PHAs with different molecular weights, as shown in Table 2.

Table 2. Molecular weight reported by PHA produced in different bacteria *(Babel and Steinbüchel et al. 2001).*

Polymer	Molecular weights [g/mol]	Ploydispersity
P3HB of *R.eutropha*	939 000 – 1400 000	1.9 – 2.25
PHA of *P.oleovorans*	178 000 – 330 000	1.8 – 2.4
PHA of *P.putida*	56 000 – 112 000	1.6 – 2.3

The properties of PHB (homopolymer), PHBV, PHB4B (scl-copolymers) and PHBHx (mcl-copolymer) are compared with polypropylene (PP) and is represented in Table 3.

Table 3. Properties of PHAs and polypropylene (PP). PHBV contains 20% 3HV-monomers, PHB4B) contains 16% 4HB-monomers, PHBHx contains 10% 3HHx-monomers *(Tsuge et al. 2002).*

Parameter	PHB	PHBV	PHB4B	PHBHx	PP
Melting temperature [ºC]	177	145	150	127	176
Glass transition temperature [ºC]	2	-1	-7	-1	-10
Crystallinity [%]	60	56	45	34	50-70
Tensile strength [MPa]	43	20	26	21	38
Extension to break [%]	5	50	444	400	400

PHA_{SCL} are typically thermoplastic polymers which can be moldable above their melting points. Melting temperature is relatively high (180 °C) and its transition temperature is between -5 and 20 ° C. This kind of PHA commonly exhibits crystallinity degree of 60 to 80% which decreases to 30 to 40% as the content of 3HV increases to 30 mol%. Incorporate 3HV into the polymer also causes T_m and T_g both decrease significantly, so short chain copolymers are more versatile materials than the homopolymer (Anderson and Dawes, 1990). As for the PHA_{MCL}, these are highly amorphous with a T_g of between -62 and -26 ° C and T_m of 42 to 58 ° C, so are classified as elastomers.

In spite of PHB being considered an environmentally friendly polymer with similar material properties to polypropylene (PP), it has not been used on a large scale to replace conventional polymers because it presents some drawbacks in its mechanical properties. Considering polymer mechanical properties, it is important to consider three basic properties when comparing the usefulness of a polymer for given the commodity application. It is hard to process PHB cause of its high melting temperature (177ºC), which it is so close to its degradation temperature. Therefore, a solution to these drawbacks could be the copolymerization of 3-HB with other monomers that confer less stiffness and tougher properties (which bestow greater flexibility and lessen breakage) and to reduce the melting point. When processing biopolymers, it is important to know the point of thermal degradation. Research recently determined that PHB decomposition starts at 246.3ºC, while the point of thermal degradation for PHBV is 260.4ºC. This indicates that the presence of valerate in the chain increases the thermal stability of the

polymer (Verlinden *et al*. 2007). The monomer composition of PHA has considerable effects on its physical properties. The mechanical properties of PHAs are directly correlated with their structure and crystallinity. An increase in the variety of side chains within one polymer chain of PHA_{MCL} can modify its ability to crystallize and as a consequence there are some distinct differences in the crystallize of PHA_{MCL}. Obtaining a low crystallinity is possible done once the polymers have large and irregular pendant side groups attached. These groups inhibit the close packing of the polymeric chains in a regular three-dimensional fashion to form a crystalline array. The physical and material properties of PHAs are greatly influenced by their monomer composition and chemical structure *i.e.* the length of the pendant groups that extend from the polymer backbone, the chemical nature of the pendant groups and the distance between the ester linkages in the polymer (Verlinden *et al*. 2007).

5.3. BIODEGRADABILITY

An important characteristic of PHAs is their biodegradability (See Appendix 1). Cause of the large size of the individual polymers prevents them to be transported through cell membrane, so the microorganism must have the ability to hydrolyze to their corresponding monomers. Microorganisms in nature are able to degrade PHAs by using PHA hydrolases and PHA depolymerases. These microorganisms degrade PHA to carbon dioxide or methane, in anaerobic or aerobic conditions, without producing toxic products. The rate of degradation of PHA, even under controlled environmental conditions is difficult to predict. Normally high temperatures allow better degradation, probably due to increased microbial activity (González-García *et al.* 2013). The polymer parameters that affect the rate of degradation include the monomeric composition of the polymer, the level of crystallinity or amorphous regions (a higher crystallinity a lower biodegradation) and molecular weight (low molecular weight polymers degrade more quickly than high molecular weight polymers). Microorganisms colonize the surface of polymers, secreting depolymerases, which hydrolyze the ester bond of the PHA producing oligomers, dimers that are subsequently taken to their monomeric forms such as P3HB to hydroxybutyrate or P3HV to hydroxyvalerate, by hydrolases. PHAs have been proved biocompatible, which means they have no toxic effects in living organisms (Bonartsev *et al.* 2007). Within mammals, the polymer is hydrolysed only slowly. PHAs are fully biodegradable to

carbon dioxide (CO_2) and water (H_2O) under aerobic conditions, and to methane (CH_4) under anaerobic conditions, by microorganisms found in soil, sea, lakes, sewage, etc. (Fig. 6)

Carbon dioxide that is released as the final mineralization product of biopolymers is used as the renewable carbon source for their biosynthesis. Photosynthetic fixation of the released carbon dioxide by plants generates renewable carbon sources again. Thus, the carbon flux in the synthesis and degradation of biopolymers is balanced. PHAs therefore do not contribute to global warming.

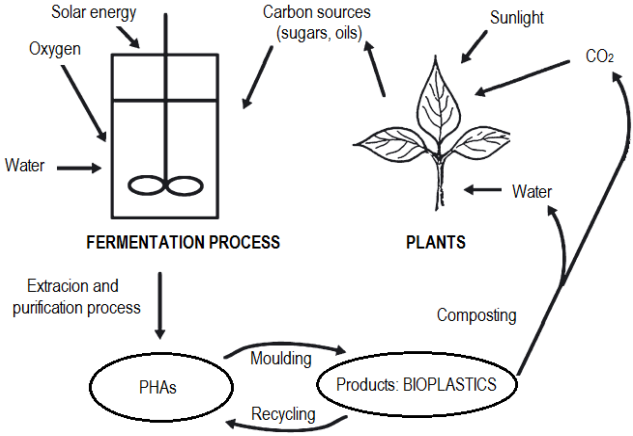

Figure 6. The life cycle of PHAs.
(Imatge from Gross and Kalra, 2002)

5.4. MATERIAL APPLICATIONS

PHA has a wide range of potential applications because of its desired features such as biocompatibility, biodegradability and negligible cytotoxicity to the cells. Hence, the potential applications of PHA as replacement for petrochemical based polymers are gaining popularity in various fields involving packaging, medical and coating materials. These desirable properties in compounding and blending have broadened their performance as potential end-use applications (González-García *et al*. 2013).

Applications focus in particular on packaging such as containers and films. In addition, their use as biodegradable personal hygiene articles such as diapers and their packaging have already been described. PHAs have also been processed into toners for printing applications and adhesives for coating applications. Composites of bioplastics are already used in electronic products, like mobile phones. Potential agricultural applications include encapsulation of seeds,

encapsulation of fertilizers for slow release, biodegradable plastic films for crop protection and biodegradable containers for hothouse facilities. It is believed that silicone, the traditionally used polymer, has maligned effects and contributes to cancer cell growth. PHA has the main advantage in the medical field, so it is a biodegradable plastic that can be insert into the human body and without the need to be removed again. PHA has an ideal biocompatibility as it is a product of cell metabolism and also 3-hydroxy butyric acid (the product of degradation) is normally present in blood at concentrations between 0.3 and 1.3 mmol l^{-1} (Verlinden *et al.* 2007). Although PHA can serve as substitute biomaterials for silicone, five key elements need to be fulfilled for successful applications of PHA in tissue engineering, *i.e.* biocompatibility, to support cell growth and cell adhesion, to guide and organize the cells, to allow cell growth and the passage of nutrients as well as waste products, and biodegradability without producing any harmful compounds. In a pure form or as composites with other materials, PHAs are used as sutures, repair patches, orthopedic pins, adhesion barriers, stents, nerve guides and bone marrow scaffolds. An interesting aspect of PHA scaffolds is the fact that the tissue-engineered cells can be implanted with the supporting scaffolds. Research shows that PHA materials can be useful in bone healing processes. Polymer implants for targeted drug delivery, an emerging medical application, can be made out of PHAs (Chen and Wu, 2005). However, because of the high level of specifications for plastics used in the human body, not every PHA can be used in medical applications. PHA used to be in contact with blood has to be bacterial endotoxins-free, which leads to high requirements in the extraction and purification methods for medical PHAs, so it means a high cost.

5.5. HISTORY ABOUT THE INVESTIGATION OF PHA

The microscopic observation of refractive granules in bacterial cells dates back to the year 1888. However, the determination of the composition of these granules was in 1925, as a result of studies of Lemoigne in *Bacillus megaterium*. In his research he found that when it degraded, this unknown material released 3-hydroxybutyric acid and subsequently described the material as a homopolyester 3-hydroxybutyrate, or poly-3-hydroxybutyrate (P3HB). During the following years, interest in these materials was limited. In 1958, Macrae and Wilkinson realized that the P3HB had a functional role when they observed that *B. megaterium* accumulated homopolymer when the ratio of the average carbon nitrogen was high, and that its degradation occurred

rapidly in the absence of an exogenous source of carbon and energy. They concluded that the P3HB was a carbon material and energy that slowed autolysis and cell death (Braunegg *et al*. 1998). From since, there was an extensive process of research on the production of this biopolymer in microorganisms outside genus *Bacillus* like *Pseudomonas, Azotobacter, Hydrogenomonas, Chromatium, Cyanobacterium* and many others. The chemical and physical properties of P3HB, as molecular weight, melt temperature, crystallinity, granule morphology and methods of extraction, identification and quantification, degradation and physiological function were also studied (Babel and Steinbüchel, 2001). The potential of P3HB for use in industrial applications was recognized in the first half of the 1960s, when the first patents related to its production through fermentation, extraction, plasticizing and mixing with other materials emerged. However the petroleum-based plastics were cheaper at that time and there was no reason to believe that fossil fuels will not be remained at a low price, because the environmental protection policies were not established at that time (Braunegg *et al*. 1998). In 1973, petroleum-based oil prices increased, which led to the conclusion that the petroleum-based oil industries could not be sustained in the future, so an intensive research to find alternative plastic products began. In 1976 the company Imperial Chemical Industries (ICI) of England began investigating whether the P3HB could be economically produced by bacterial fermentation of carbohydrates from agriculture. In the 1980s, Wallen and Davis first reported the isolation of a biopoliester with physical properties similar to P3HB but with a different chemical composition. Later, analyzes revealed the presence of 3-hydroxyvalerate (3HV), 3-hydroxybutyrate, 3-hydroxyhexanoate (3HHx) and 3-hydroxyheptanoate (3HHp). This was the first report of a heteropolymer, and because of the diversity of hydroxyalkanoic acid constituents these biopolymers (including P3HB) began to be called generally polyhydroxyalkanoates (PHA).

Currently, the PHA longer produced commercially (González-García *et al*. 2013). Among the most important there are the copolymer of 3HB and 3HV. Many companies have produced or are currently producing them. The most important are shown in Appendix 2.

6. PRODUCTION OF PHA

Nowadays, the main candidates for the large-scale production of PHAs are plants and bacteria. Plant cells would be the ideal alternative for the production of biopolymers due to the possibility of cultivating them in large quantities using the most economical source of carbon: sunlight. However, plant cells can only cope with low yields (<10% of dry weight) of PHA production because high levels (10–40% of dry weight) of polymer inside the plant have a negative effect on the growth and development of the plant. At present, this problem has not been overcome. In contrast, within bacteria, PHAs are accumulated to levels as high as 90% of the dry cell mass. Accumulating PHAs is a natural way for bacteria to store carbon and energy, when nutrient supplies are imbalanced. These polyesters are accumulated when bacterial growth is limited by depletion of nitrogen, phosphorous or oxygen and an excess of a carbon source is still present. While the most common limitation is nitrogen, for some bacteria, the most effective limitation is oxygen. As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside the cells. It is advantageous for bacteria to store excess nutrients inside their cells, especially as their general physiological fitness is not affected. The surface of a PHA granule is coated with a layer of phospholipids and proteins. Phasins, a class of proteins, are the predominant compounds in the interface of a granule. The phasins influence the number and size of PHA granules (Verlinden *et al*. 2007).

Effectiveness of MMC PHA production processes is strongly dependent on culture selection by the conditions imposed on the bioreactor. In principle, the feast-famine (FF) regime is used to achieve the goal. The need to impose FF conditions limits the organic load and, thus, most of the MMC PHA production studies are usually operated in two separate stages: (1) the culture selection and (2) the PHA accumulation. The physical separation of the culture selection and PHA production stages allows for the optimization of the process, as different optimal conditions were shown to be required in each stage. Moreover, as most of the available agro-industrial waste/surplus feedstock cannot be directly converted to PHA (MMCs do not store carbohydrates as PHA but rather as glycogen). PHA production by mixed cultures from waste- and surplus-based feedstock requires a previous anaerobic fermentation stage to convert their organic content into VFAs. Thus, the resulting process usually comprises three stages (Fig. 7):

(1) the acidogenic fermentation stage, (2) the culture selection stage, and (3) the PHAs production stage (Reis *et al*. 2011).

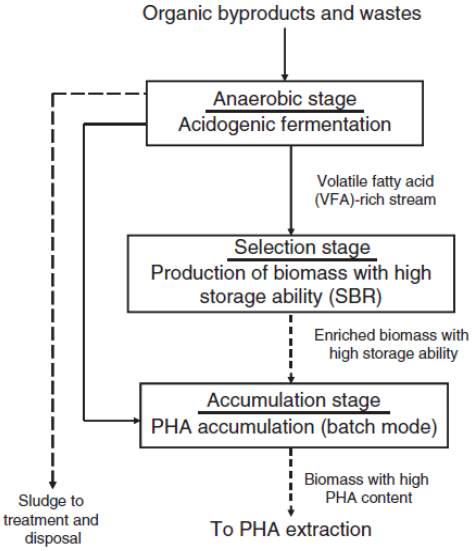

Figure 7. Schematic representation of a three-stage sequential process for PHA production from waste by mixed cultures using feast and famine sequencing batch reactor (SBR).

(Image from Reis et al. 2011)

6.1. RAW MATERIALS

Cost of raw materials, mainly carbon sources, is the one of the most important factors affecting the overall economics of PHAs production, specifically for large-scale, without taking account the recovery process (Amache *et al*. 2013). Thus, the selection of suitable carbon substrate is a critical factor that determines the overall performance of the bacterial fermentation and the cost of the final product. Therefore, the simplest approach is to choose renewable, inexpensive and most readily available carbon substrates that could support both the microbial growth and PHA production efficiently. Microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive, complex waste effluents to plant oils, fatty acids, alkanes and as well as simple carbohydrates. So, three types of renewable feedstock are available for PHA production: grains or their components, agriculture and forestry biomass, and industrial wastes. Each year, a large amount of waste materials are discharged from agricultural and food processing industries and these wastes represent a potential renewable feedstock for

PHA production. Utilizing these waste materials as carbon source for PHAs production not only reduces the substrate cost, but also saves the cost of waste disposal. The most common waste, inexpensive carbon source used as an industrial waste material is molasses, either from sugar cane or beet (Salmiati *et al*. 2007 and Koller *et al.* 2012). Various strains have been evaluated for their capability to produce PHAs from beet molasses and sugar cane. Organic wastes discharged from industrial processes, such as molasses and cheese whey, are readily available for PHA fermentation. Some type of pretreatment may be necessary before the waste stream can be utilized by microbial PHA-producers. Whey is the residual watery portion of milk from cheese manufacturing (Chanprateep *et al*. 2010). The opaque liquid contains about 6% solids and has a high biological oxygen demand.

Carbon dioxide is the most abundant source of carbon in the ecosystem, and for this reason, is an interesting substrate cause of it has lots of availability. Plants use carbon dioxide and sunlight as food, so they are used as a carbon source in the fermentation process. Similar to plants, cyanobacteria also pose as attractive PHA producers that utilize CO_2 and sunlight as carbon and energy sources. Cyanobacteria are oxygen-evolving photosynthetic bacteria that naturally possess the key enzyme for the production of PHA, which is the PHA synthase.

6.1.1. Available waste streams in different global regions

As mentioned above, the main drawback of the large-scale production of PHA is the high cost of raw materials. Thus, a viable solution strategy is identified by the utilization of a broad range of waste and surplus materials that can be upgraded to the role of feedstock for the biomediated production of desired end products such as polyhydroxyalkanoates biopolymers. The selection of the appropriate waste stream as a feedstock for biotechnological purposes mainly depends on the global region where the production plant will be constructed (Jiang *et al.* 2011). To save costs for transportation, facilities for the production of biopolymers, biofuels and biochemicals should be integrated into existing production lines, where the feedstock directly accrue as waste streams (Chen *et al*. 2009). In addition to their main components, complex waste streams can contain additional substances that make them advantageous in direct comparison to pure and expensive substrates. For example, the permeate of surplus whey from the dairy industry provides the production strain in bioprocesses not only with a rich source of

the carbohydrate lactose, but also with minor components such as minerals and protein residues that have positive impacts on the microbial cultivation (Koller *et al*. 2010).

6.1.1.1. Waste lipids

Several waste lipids of different origin can be applied as substrates PHA production:

(a) Waste cooking oil and restaurant greases are waste products available in large amounts.

(b) Tallow from the slaughtering and rendering industries constitutes another inexpensive source of triacylglycerides.

(c) In PHA production, biomass has to be degreased before isolation of PHA if high product purities are required. Also here, typically 2–4% of lipids are removed from the cells.

6.1.1.2. Waste streams from Biofuel production

The production of biodiesel normally is accomplished from different lipids such as food-grade rape seed oil, or palm oil. Today, the utilization of food-grade raw materials for combustion is neither economically feasible nor acceptable from an ethical point of view. As an alternative, lipid wastes such as used cooking oil, restaurant greases and soapstocks are valuable feedstock for cost-efficient biodiesel production (Vasudevan and Briggs, 2008).

Glycerol is generated in bulk particularly from the co-product stream of biodiesel, and thus possesses great potential to be an attractive carbon source for PHA production by certain microorganisms (Ashby *et al*. 2005). Together with rising costs for petrol-based fuels, the production of biofuels is increasing enormously in many areas of the world, consequently decreasing the price of the by-product glycerol (Koller *et al*., 2010 and Moralejo-Gárate *et al*. 2011).

6.1.1.3. Surplus Whey from the Dairy Industry

Whey from the dairy and cheese industries constitutes a waste and surplus material in many regions of the world. It is not only a cheap raw material, but also causes a disposal problem for the dairy industry owing to its high biochemical oxygen demand and chemical oxygen demand (Kim *et al.* 1995). Lactose, the major carbohydrate in whey, can serve as a substrate for growth and product formation in numerous biotechnological processes such as PHA production (Koller *et al.* 2010).

6.1.1.4. Lignocellulosic Wastes

Industrial branches generating the major shares of this waste are the agro-industry, the wood-processing industry and the paper industry. The selection of appropriate production strains for PHA biosynthesis from lignocellulose-derived substrates mainly depends on the conversion rates of hexoses and pentoses by the organism. If the different sugars are not used in parallel and with similar rates, the bioprocess development will be rather complicated. Sugars that are not accepted as a substrate by the strain or that are utilized considerably more slowly than others can pile up in the fermentation broth and may then cause inhibitory effects that are very likely to negatively influence growth and production kinetics and yields (Kumar *et al*. 2008). A prime example for this can be found at the company PHBISA in Brazil, where, starting from sugar cane, sucrose, bioethanol and PHA biopolyesters are produced. The energy required for these processes is totally supplied from burning of bagasse that accrues in high amounts as a residue from the sugar cane plant.

6.1.1.5. Materials from the sugars industry

A different approach is provided by the utilization of carbon sources that have a considerable market value and do not constitute waste materials, but are produced in a process integrating the fabrication of the carbon substrate and PHA. This has been implemented on a pilot scale by the company PHB Industrial in the state of Sao Paulo, Brazil. Starting from sugar cane, the company produces sucrose and ethanol. The waste streams from the sugar production (bagasse) and the bioethanol production (fusel alcohols) are used for running the PHA production and making it economically competitive (Koller *et al*. 2008).

6.2. METABOLIC ROUTES OF PHA BIOSYNTHESIS

PHA can be synthesized either by chemical means or by biological approaches. Biosynthesis of PHA leads to much a higher molecular weight compared with that achieved with chemical methods. However, biosynthesis of PHA does not allow much control over the monomer structures in the PHA polymers; the specificity of PHA polymerase (or PHA synthase) will influence the monomers incorporated into the polymers. Since biosynthesis of PHA is conducted by microorganisms grown in an aqueous solution containing sustainable resources such as starch, glucose, sucrose, fatty acids, and even nutrients in waste water under 30–37 °C and atmosphere pressure. This fact is considered as more environmentally friendly and

sustainable, especially when petroleum as a non-sustainable resource is being depleted quickly. PHA biosynthesis has been studied over the past many years. Acetyl-CoA is the key components to supply the 3-hydroxyalkanoatyl-CoA of different lengths as substrates for PHA syntheses of various specificities. In addition, 3-hydroxyalkanoyl-CoA can also be supplied from β-oxidation of fatty acids of different chain lengths. Many genes encoding various enzymes are directly or indirectly involved in PHA synthesis. The bacteria produce different PHA depending on the length of the hydrocarbon chain between the carboxyl group and the side chain R, and the length of the side chain itself. When the organism is growing in normal conditions and there is enough free coenzyme A, it favors the formation of acetyl-CoA and its entry into the TCA cycle to produce energy. However, when the entry of acetyl-CoA in the TCA cycle is inhibited, by limiting nutrients (mostly nitrogen), its excess is utilized for the synthesis of PHA. Thus, when the limiting nutrient stops being and / or decreases the carbon source in the medium, the cell used as reserve the PHA contained in cytoplasm granule by the activation of the intracellular PHA-despolymerase (PhaZ). Thanks to this enzyme the process can be reversible, generating acetyl-CoA which enters the TCA cycle to produce energy (Reis *et al*. 2011).

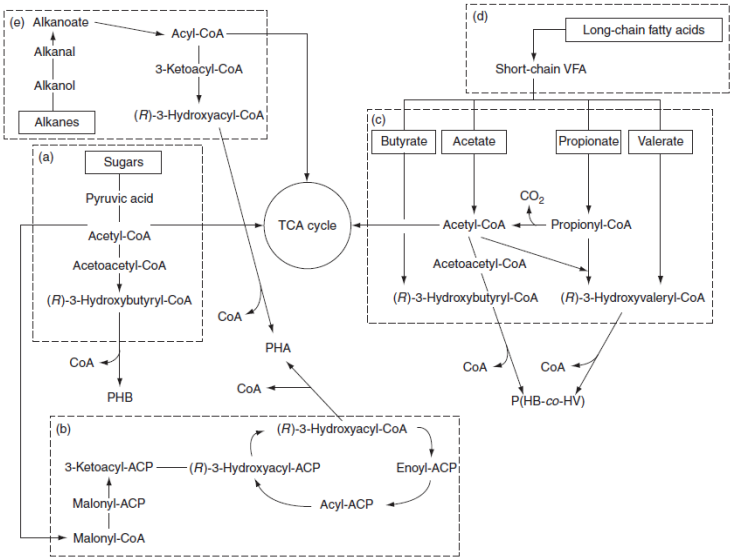

Figure 8. Metabolic pathways involved in PHA synthesis from sugars, through glycolysis (a) or de novo fatty acid biosynthesis (b); from fatty acids, directly (c) or through fatty acid β-oxidation (d); and from alkanes, through alkane oxidation (e). (*Image from Reis et al. 2011)*

6.3. BACTERIAL STRAINS

PHA can be produced by using pure cultures of bacteria or mixed cultures (Table 5). In any case, when PHA is produced from waste, it works in mixed culture of bacteria, and it is really difficult to know exactly what type of bacteria there is present and what not. However, when PHA is produced from pure cultures, PHAs are produced by many different bacterial cultures. Among the more than 250 different natural PHA-producers, only a few bacteria have been employed for the biosynthesis of PHA. *Cupriavidus necator* is the one that has been most extensively studied. A few important other strains that were recently studied include: *Bacillus spp.*, *Alcaligenes spp.*, *Pseudomonas spp.*, *Aeromonas hydrophila*, *Rhodopseudomonas palustris*, *Escherichia coli*, *Burkholderia sacchari* and *Halomonas boliviensis*. An overview of bacterial strains used to produce PHAs is given in Appendix 3.

Table 4. Comparison between pure culture and mixed culture.

Pure culture	Mixed culture
High PHA yields	Relative low PHA yields
High volumetric productivities	Relative low volumetric productivities
High operation cost	Lower operational costs – no sterilization and less control required
High investment	Make easier the use of waste organic substrates

6.4. EXPERIMENTAL STRATEGIES FOR PHA PRODUCTION

A lot of batteries have been investigated to produce PHA, mainly short-chain-length bacteria. Depending on the culture conditions that favor PHA accumulation, bacteria that are used for the production of PHA can be classified into two groups. The first group of bacteria requires limitation of essential nutrient (nitrogen, oxygen, phosphorus) and presence of excess carbon source for the efficient synthesis of PHA. The second group of bacteria does not require nutrient limitation for PHA production, and can accumulate PHA during the exponential growth phase (Johnson *et al.* 2009). Overall, the profitability of a bacterium to produce PHA industrial scale depends on several factors such as: the stability of the organism, the speed of accumulation of the polymer, the rate of growth, cell density that can achieve the polymer

content, ease of extraction, molecular weight of the polymer, the possible range of usable carbon sources, the generating byproducts and the cost of the medium.

The culture conditions required for PHA production are important criteria to be taken into consideration for the development of cultivation techniques used in the large scale production of PHA. Batch and fed-batch fermentation are widely used in the industrial fermentation processes. Batch fermentation for PHA production is a popular process due to its flexibility and low operation costs. However, it is associated with low PHA productivity since after bacterial cells degrade the accumulated PHA resulting in reduced PHA content. Fed-batch is more efficient than batch cultivation in terms of achieving high product and cell concentration because the medium composition can be controlled by substrate inhibition. Batch and fed-batch processes are thus combined as a result of low PHA content obtained by each process individually. The combined process is the most common fermentation strategy used for PHA production. Under this strategy, the process is divided into two stages: in the first one the microorganism is grown under batch mode until the desired biomass is achieved without nutrient limitation and PHA accumulation has started. In the second stage the fermentation is shifted to fed-batch, where usually one or more essential nutrients (most common is nitrogen) are maintained in limited concentration and carbon source is continuously fed into the reactor to produce and accumulate PHA in the cells. During this nutrient limitation stage, cells are unable to multiply and remain almost constant. However, cells begin to increase in size and weight due to the intracellular accumulation of PHA as a storage product. This process is known like a *feast- famine* process (Reis *et al.* 2011).

Another strategy to produce PHA, considering like a third method, is a continuous culture, it means, chemostat. In this method the culture broth is continuously replaced by sterile medium. The carbon source is continuously fed in excess, keeping one or more nutrients (phosphorous or nitrogen) in limitation. This method is highly controllable under appropriate growth conditions; continuous fermentation might have the potential to give highest PHA productivity levels (Amache *et al*. 2013).

6.4.1. Operation methods

The choice of operation methods strategy for production of PHA depends on various factors including carbon source (glucose or complex waste material), culture (pure or mix), mode of fermentation (batch, fed-batch, continuous) or bioreactor type (Amache *et al*. 2013). The

fermentation may be carry out in a single stage or multi stages of sequencing batch system (SBR). Mixed cultures are microbial populations operating in open biological systems, whose composition depends directly on the substrate mixture and operational conditions imposed on the bioreactor. These have been used for decades in biological wastewater-treatment processes. Activated sludge systems, including enhanced biological phosphorus removal systems, were shown to select for PHA-accumulating organisms when operated under dynamic feeding conditions (Valentino *et al*. 2014).

6.4.1.1. Feast and famine process: Fully aerobic or anaerobic/aerobic

Synthesis of PHA by mixed cultures was first observed in wastewater treatment plants (WWTP) designed for biological phosphorus removal. These systems are operated with alternated anaerobic and aerobic cycles. Activated sludge with significant PHA storage capacity was also observed in aerobic wastewater-treatment plants and this process configuration creates periods of excess and lack of external carbon substrate. The enhanced PHA storage demonstrated by microbial cultures operated under feast and famine (FF) conditions results from the internal growth limitation caused by transient substrate availability. Following a certain period of time in the absence of external carbon substrate, a decrease of the amount of intracellular components required for cell growth (RNA and enzymes) occurs. Therefore, when carbon substrate becomes available again, the amount of growth enzymes may not be enough to ensure that a maximum growth rate is reached (Daiger and Grady, 1982). On the contrary, fewer enzymes are required for PHA storage and, therefore, PHA storage can occur at a much faster rate than cell growth, thus providing the cells with a means of rapidly consuming the available external substrate. The need for physiological adaptation, following each starvation period, is considered the main mechanism triggering for PHA storage by microorganisms subjected to FF conditions (Beccari *et al.* 1998). Fully aerobic activated sludge cultures, following long periods of starvation, were shown to channel more carbon toward storage than toward growth and maintenance (Reis *et al.* 2011). After the external carbon substrate is depleted, the internal PHA reserves serve as carbon and energy sources for cell growth and maintenance. In this way, a competitive advantage exists for microorganisms that are quicker to store the substrate and reuse it for growth. Thus, operating conditions and substrates that select for PHA storage also cause an enrichment in the mixed microbial cultures of best storing microorganisms. The imposition of transient external substrate availability is currently known as

'aerobic dynamic feeding' (ADF) or FF regime. This allows for the selection of cultures with a high and stable capacity of PHA production during feast and good capacity to grow on PHA during famine phase. PHA storage is also observed in Enhanced Biological Phosphorus Removal (EBPR) systems, which are operated with alternating anaerobic/aerobic cycles. In anaerobic/aerobic cycles (Fig. 9 (a)) the external carbon substrate is only available in the absence of the final electron acceptor (anaerobic period), thus limiting the possibility of growth during this period. Only in the subsequent aerobic stage can the cells grow – using the then available oxygen for oxidative phosphorylation – however, at this stage, the external substrate has already been depleted. Just as for fully aerobic FF (Fig. 9 (b)), the transient availability of carbon substrate, and consequent internal growth limitation, creates a strong pressure for PHA storage.

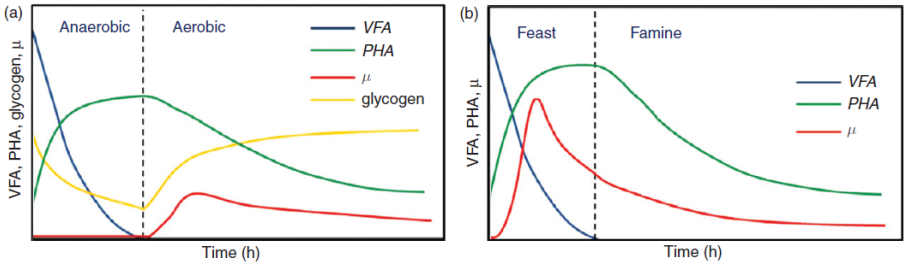

Figure 9. Operation methods for PHA storage by mixed microbial cultures under (a) anaerobic/aerobic feast and famine and (b) fully aerobic feast and famine.

(Image from Reis et al. 2011)

The two main differences between anaerobic/aerobic feast-famine and fully aerobic feast-famine are:

a) Cell growth rate: in anaerobic/aerobic FF, cell growth occurs only from the intracellular polymer, and so the observed growth rate is typically lowers that in ADF, in which cell growth can occur from the external substrate concomitantly with PHA storage.

b) Polymer composition, due to the presence of a second storage compound (glycogen) whose degradation can serve to produce precursors for PHA synthesis.

In general, anaerobic/aerobic FF systems select for cultures with lower productivities than those reported for ADF systems, but with a broader range of polymer compositions (Serafim *et al.* 2008)

6.4.2. Stages of production process

In this work a piloting prototype has been created to produce PHA from wastewater. The fermentation process is divided into three stages (Fig. 10):

(a) Stage 1: VFA-rich concentrate production from a fermentation process.

(b) Stage 2: produce of biomass with increased PAP by treating the influent municipal wastewater.

(c) Stage 3: produce PHA-rich biomass by exploiting the PAP of the harvested surplus biomass from stage 2 when fed with centrate from stage 1.

6.4.2.1. Stage 1: VFA-rich concentrate production from a fermentation process

The acidogenic fermentation is the initial phase of the anaerobic digestion of organic compounds to methane and carbon dioxide: soluble organic compounds are fermented into organic acids, such as acetic, propionic, butyric, and lactic acid, and other fermentation products, such as alcohols and hydrogen (Reis *et al.* 2011). This stage comprises a control system to control the temperature, a stainless stirred tank and a downstream centrifugation unit to separate solids. The acidogenic fermentation is fed with waste activated sludge (WAS) without primary treatment (Tamis *et al.* 2014). The operation mode is in batch-wise without pH control, wherein inherent buffering kept pH between 5.5 and 6.5 or adding a NaOH solution (Tamis *et al*. 2014 and Morgan–Sagastume *et al.* 2015). Studies have been done in a fermentation reactor with mesophilic and thermophilic conditions in order to determine the best temperature so it gets better performance. Through some experiments, the best performance was obtained at 42ºC with batch-fermentation times of 4-5 days to produced centrate as feedstock for stage 3 (Appendix 6). To keep the reactor effluent nitrogen depleted (favorable for use in the accumulation reactor later in the process) the target COD:N mass ratio must be around 300:1. The volatile fatty acids (VFA) obtained in centrifuge unit are use like a feedstock for the stage 3. The other stream in the centrifuge unit is the cake (solids). This byproduct is conducted to an anaerobic digestion (AD) in order to treat this waste. Two products are obtained from AD: biogas and digestive. Biogas produced during anaerobic digestion is primarily composed of methane (CH_4) and carbon dioxide (CO_2), with smaller amounts of hydrogen sulfide (H_2S) and ammonia (NH_3). Trace amounts of hydrogen (H_2), nitrogen (N_2),

carbon monoxide (CO), saturated or halogenated carbohydrates and oxygen (O_2) are occasionally present in the biogas (Michael *et al.* 2003).

Table 5. Composition of waste activated sludge (WAS) in acidogenic fermentation. *(Morgan-Sagastume et al. 2015)*

Parameter	Full name
TS	Total solids
VS	Volatile solids
COD	Chemical oxygen demand
COD_{SOL}	Soluble chemical oxygen demand
COD_{VFA}	Volatile fatty acids in COD
N_{total}	Total nitrogen
NH_4^+	Ammonium
P	Phosphorous

Each author uses a different feed medium and a different nutrient source. Appendix 4 shows a table with the values of each component.

The different working methods of each author in this first stage of fermentation are shown in Table 6.

Table 6. Material and methods at the stage of fermentation.

FERMENTATION REACTOR									
V [L]	**O.M.**	**T** [ºC]	**pH**	**pH control**	**Cycle length**	**HRT**	**SRT**	**COD: N**	**Reference**
60	In series	(1) 30±0.1 (2) 40±1	4.5± 0.1	1M NaOH	*	4 h	4 d	300:1	Tamis *et al.* 2014
*	C.F.	30	6	2M NaOH	*	16h	*	*	Benstsson *et al.* 2008
1.8	Batch-wise	30	*	No	12h	14h	24h	*	Palmeiro-Sánchez *et al.* 2014
1000	Batch-wise	35 42 55	5.5 – 6.5	No	*	*	*	100:2	Morgan-Sagastume *et al*. 2015

3	Parallel semi-continuous	(1) 42 (2) 55	6.1 – 6.6	*	*	1 - 6	1 – 6	*	Karlsson *et al*. 2010

O.M.: Operation method; C.F.: Continuous flow; (1): 1st reactor; (2): 2nd reactor; (*) Information not available.

6.4.2.2. Stage 2: produce of biomass with increased PAP by treating the influent municipal wastewater

The first aim of the selection stage is to obtain from activated sludge a culture highly enriched in organisms that are highly adapted to FF, and so with high and stable PHA storage capacity. Microorganisms presenting low storage capacities would have a negative impact not only on the productivity of the final accumulation stage but also on the downstream processing, thus increasing the PHA extraction costs. In most cases, this stage takes place in a SBR under feast-famine regime. According to Morgan-Sagastume *et al.* (2015), this step consist of a wastewater delivery line including a primary treatment with a drum microfilter in order to remove solid, a feed-holding tank to put the municipal wastewater treatment, a sequencing batch reactor and aerated tank for active biomass storage (Fig. 10). This stage is fed from municipal wastewater influent after screening, fat and sand removal (Morgan-Sagastume *et al*. 2015). The SBR treating the filtered influent municipal wastewater operated under aerobic feast-famine (Johnson *et al.* 2009) at high organic loading rates (OLR) and a short hydraulic retention times (HRT).

The wastewater delivery line and the drum filter should be purged in order to ensure a good representative influent feed in stage 2. The stage 2 operation as an SBR is meant to mimic an idealized continuous flow process with a short aerobic (feast) plug-flow contact volume, followed by solids separation, and then a plug-flow aerobic (famine) unit process for the return activated sludge biomass (Fig. 10). The SBR is operated with total suspended solids (TSS) control in order to sustain a stable specific OLR. There is not a temperature control in SBR, thus it is operated within the full impact of seasonal temperature variations. The tank for biomass storage is constantly aerated to supply oxygen to the system and ensure no oxygen limitation occurred.

The efficiency of the culture selection stage is determinant for the PHA accumulation performance obtained in the subsequent production stage.

Just as in the previous section, the following table shows the different methods and characteristics used by different authors at this stage of enrichment.

Table 7. Materials and methods at the stage of enrichment biomass.

ENRICHMENT REACTOR										
V [L]	**O.M.**	**Cycle length**	**HRT**	**SRT**	**T** [ºC]	**Air flow** [L/min]	**pH**	**pH control**	**ATU**	**Ref.**
200	SBR	12h	24h	24h	30±2	200	6.5 – 7.5	1M HCl and 1M NaOH	10 -20 mL/L [33g/L]	Tamis *et al.* 2014
2	SBR	24 h	48h	48h	30	*	7	*	1.5 ml/L [33g/L]	Moralejo-Gárate *et al.* 2014
2	C.F.	*	*	7d	30	*	7.3	2M HCl	*	Bengtsson *et al.* 2008
1.8	SBR	12h	24h	24h	30	*	9.2 ±0 .4	No	1.5mL/ L [33g/L]	Palmeiro-Sánchez *et al.* 2014
500	SBR	2h	3h	*	8.4 – 22.8	66.6 – 83.3	5.6 – 6.4	No	*	Morgan-Sagastume *et al.* 2015
2	SBR	12h	1d	1d	30	*	7.0 ±0 .05	1M HCl 1M NaOH	100 mg/L	Jiang *et al.* 2011
2	SBR	12h	1d	1d	30±1	1.3-1.4	7.0 ±0 .1	1M HCl 1M NaOH	100 mg/L	Johnson *et al.* 2009
0.75	2- P. SBR	4h	3d	3d	35	*	*	*	*	Karlsson *et al.* 2010
2	SBR	6h	48h	48h	30	1.0 – 1.1	7.0 ±0 .1	0.5M NaOH 0.5M HCl	1.5 mL/L [33g/L]	Moralejo-Gárate *et al.* 2013
2	SBR	24	48h	48h	30	1	7.0 ±0 .1	0.5M NaOH 0.5M HCl	1.5 mL/L [33g/L]	Moralejo-Gárate *et al.* 2011
2	SBR	12h	1d	1d	30	0.2	7	1M HCl 1M NaOH	5mg/L	Margang *et al.* 2011

O.M.: Operation method; 2-P.: Two parallel; C.F.: Continuous flow; (*) Information not available.

Majone *et al.* (2006) demonstrated the importance of this step in the process. He compared the storage response of an activated sludge with that of an enriched culture selected under FF in an SBR. The results that he obtained are shown in the following table.

Table 8. Compared the storage response of an activated sludge with that of an enriched culture selected under FF in a SBR.

	Activated sludge	Enriched culture
PHA storage rate [mg PHA·X^{-1}·h^{-1}]	21	405
Polymer content [%]	10	44

This difference was explained by showing that most bacterial components of the enriched culture were responsible for PHA storage, whereas only a small fraction of the microorganisms in the activated sludge was able to store PHA.

6.4.2.3. Stage 3: produce PHA-rich biomass by exploiting the PAP of the harvested surplus biomass from stage 2 when fed with centrate from stage 1.

This stage consists in to get the maximum storage capacity of the mixed culture PHA previously enriched. Stage 3 comprises an accumulation reactor, a downstream active biomass unit, a dewatering centrifuge and a dry oven (Fig. 10) (Morgan-Sagastume *et al.* 2015). A small pulse addition of substrate (with composition identical to the nutrient solution used for the fermentation) is provided when the dissolved oxygen levels in the reactor start to increase as a response to substrate consumption. The PHA-rich biomass is dewatered and processed according appropriate methods.

Table 9. Materials and methods at the stage of accumulation reactor.

ACCUMULATION REACTOR							
V [L]	**O.M.**	**N.L.**	**T** [ºC]	**pH**	**pH control**	**COD:N:P or COD/N**	**Reference**
200	Feed-batch	N	30±2	6.5 – 7.5	1M HCl and 1M NaOH	*	Tamis *et al*. 2014
2	Feed-batch	N	30	7	*	*	Moralejo-Gárate *et al.* 2014
*	*	N&P	30	7.3±0.2	*	100:0.030:0.0015	Bengtsson *et al.* 2008
*	*	N	30	-	No	*	Palmeiro-Sánchez *et al.* 2014
550	Feed-batch	N&P	28	7.3 – 8.4	*	100:1:0.05	Morgan-Sagastume *et al*. 2015

2	Feed-batch	N	*	*	*	*	Jiang *et al.* 2011
1	Feed-batch	N	30±1	7±0.1	1.5M Acetic acid 1M NaOH	*	Johnson *et al.* 2009
0.48	Feed-batch	Ø	35	6.3	NaOH	*	Karlsson *et al.* 2010
*	*	*	30	7.0±0.1	0.5 M NaOH 0.5 M HCl	76 and 770	Moralejo-Gárate *et al.* 2013
2	*	N	30	7	1.5M solution of lactic acid and/or acetic acid	*	Margang *et al.* 2011

O.M.: Operation method; N.L.: Nutrient limited; Ø: Without limitation; (*) Information not available.

The strategy employed in this step is based on forcing the conversion of substrate into biopolimer preventing bacterial growth. This is achieved by limiting the presence of an essential nutrient (nitrogen, oxygen, phosphorus, etc.).

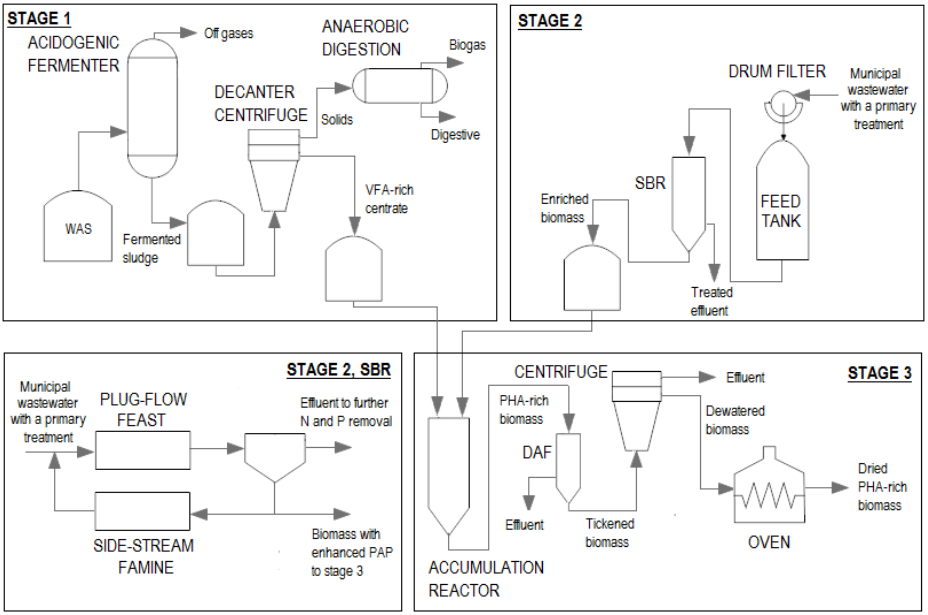

Figure 10. Schematic representation of the flow diagram of the PHA production with municipal wastewater and sludge treatment. *(Image from Morgan-Sagastume, 2015)*

Given the above scheme, the PHA production process it has been carried out also considering a recirculation in the accumulation reactor. A stream from this reactor goes into a settler tank in order to separate the effluent from the main stream which is driven to the accumulation reactor again (Morgan-Sagastume *et al.* 2015).

In Appendix 5, different schemes for the production of PHAs from waste and wastewater are shown.

6.4.3. Downstream process: PHA recovery and purification

After fermentation, bacterial cells containing PHAs are separated from the medium by centrifugation. The method applicable for the effective separation of PHA from other biomass components can be complex and costly. The most common method for the extraction of PHA from biomass is solvent extraction by using chloroform (Chee *et al.* 2010). PHA resulting solution is filtered to remove cell debris, then it is concentrated and the PHA precipitated in methanol or ethanol. With this technique, the lipids with low molecular weight remain in solution and do not interfere with the determination. By using this method, highly purified PHA can be obtained without the degradation of PHA molecules, so it is a good method for medical applications. Other halogenated hydrocarbon solvents such as dichloromethane, dichlorethane and chloropropane can be also used to extract and purify PHA from the cell biomass. However, the necessity of large quantities of solvent makes the procedure economically and environmentally unattractive. Other effective procedures have been developed to recover PHA, such as the cell lysis by using sodium hypochlorite. In this method, the cell biomass is initially treated with sodium hypochlorite solution before the PHA granules are isolated from the cell debris by centrifugation. The use of this chemical product to extract PHA from biomass always results in severe degradation of PHA and yields PHA with a lower molecular weight. In contrast, the use of surfactant pretreatment to recover PHA resulted in lower purity but less degradation of molecule thus, more performance. In order to obtain a PHA with a high degree of purity, the use of combinations of sodium hypochlorite solution with a surfactant pretreatment is carried out. Furthermore, it has been observed that reduces degradation of the molecule by dispersing a solution of sodium hypochlorite and chloroform (Chee *et al*. 2010).

A method without chemical products is the enzymatic digestion. This technique is a gentle, yet selective separation method. Enzymes such as protease, cellulose and lysozyme, are commonly used in this method. The reaction of enzymes is specific and requires only a mild operational condition for high reaction rates with little product damage; it means that this method reduces the degradation of molecule. Before the enzymatic treatment, a short period of heat shock treatment is applied to the culture broth in order to rupture the cells as well as denature and facilitate the extraction process. In addition, the efficiency of enzymatic treatment can be increased with the aid of several chemicals such as proteinase K, AlcalaseR, sodium dodecyl sulfate (SDS) or ethylene diamine tetra acetic acid (EDTA).

Finally, due to the drawback of organic solvent extraction, much attention has been given to centrifugal fractionation, a simple and economical process for separating specific resin components from the recovered PHA. The crop has to be pretreated with hexane in order to eliminate molecular lipids and oil; and ethanol (40% water/60% ethanol) to eliminate soluble compounds such as sugars. Up to 85% of PHA with purity higher than 95% can be obtained from continuous centrifugal fractionation. A part from centrifugal fractionation, recovery of PHA from crops using air classification has also been investigated. This process involves the separation of finely ground solid particles based on weight or size. The finer fractions accomplished at the end of the process will have higher PHA concentration and PHA can be recovered from the particles by any other convenient method such as filtration and centrifugation. Through this method it can be obtain up to 90% of PHA with purity within the range of 85–95% (Verlinden *et al*. 2007 and Chee *et al*. 2010).

Table 10. Extraction methods of PHA. *(Posada et al. 2011)*

EXTRACTION METHOD	ADVANTAGE	DISADVANTATGE	RESUSLTS [% w]
Extraction with solvents	Removal of endotoxins/high purity. There is not polymer degradation	Break the morphology PHA granules. High price/low recovery	Purity: 99.5 % Recovery > 90 %
Surfactants digestion	Treatment of high cell densities	Low purity/polymer degradation	Purity > 95 % Release rate > 90 %
NaClO digestion	High purity	Polymer degradation	Purity: 99 % Recovery: 94 %
NaClO and chloroform digestion	Low polymer degradation of high purity	It requires high amounts of solvent	Purity: 97 % Recovery: 91 %
NaClO and surfactants digestion	Degradation limited/low operating costs	-	Purity: 98 % Recovery: 86.6 %

Surfactants and chelates digestion	High purity/low environmental pollution	Large volumes of wastewater	Purity: 98.7 % Recovery: 93.3%
Enzymatic digestion	Good recovery	High costs of enzymes	Purity: 92.6 % Recovery: 90 %
High pressure homogenization	It does not require the use chemicals	Poor disruption/low speed disruption	Purity: 98 % Yield: 98 %
Classification aeration	High purity	Low recovery	Purity: 97 % Yield: 90 %.
Dissolved air flotation	It does not require the use chemicals	It requires many consecutive steps floating	Purity: 86%

7. REPORTED RESULTS IN LITERATURE

The results reported in this section are the outcomes from different academic articles that have worked and studied the process of PHA production from waste and wastewater.

7.1. WASTEWATER AND FERMENTATION: FERMENTED SLUDGE CENTRATE AS SOURCE OF VFAs FOR PHAs PRODUCTION

Morgan-Sagastume *et al*. (2015) and coworkers studied a batch fermentation of full-scale WWTP sludge at selected temperatures (35ºC, 42ºC and 55ºC). He designed a pilot process that supported integrated PHA production with municipal wastewater treatment (Fig. 10). Moreover, Tamis *et al.* (2014) and Palmerior-Sánchez *et al*. (2014) also produced PHA, but they did so as from industrial wastewater. Hence, in this section, the results that they obtained are discussed and compared between them.

According to Morgan-Sagastume *et al.* (2015), the full-scale waste sludge acidogenic fermentation was readily accomplished for both mesophilic (35ºC and 42 ºC) and thermophilic (55 ºC) conditions. Fermentation at 42 ºC was preferred for producing centrate as feedstock for stage 3 due to the higher VFA yields and VFA conversions of soluble COD at this temperature. Nevertheless, WAS fermentation at 35ºC and 55 ºC also produced VFA-rich streams amenable for the use in stage 3. Even 30 ºC has been found to be a practical temperature for sludge fermentation (Pittmann and Steinmetz, 2013). In a temperature of 55 ° C was observed at low

COD_{sol} conversions into VFAs. Comparing the performance of VFA, it was deduced that the lack of primary treatment in WWTP may have provided a more fermentable waste sludge. However, primary sludge and primary-WAS mixtures tend to be more fermentable and likely more suitable for VFA production (Pittmann and Steinmetz, 2013).

The maximum VFA levels ($gCOD_{VFA}/L$) that were achieved in their study are: 6.0 ± 0.9 at 35 ºC; 7.0 ± 1.0 and 9.4 ± 1.6 at 42 ºC; 7.1 ± 1.9 at 55 ºC (Morgan-Sagastume *et al.* 2015), and the mesophilic and thermophilic fermentation products were consistently dominated by acetic (28– 38%, COD basis), butyric (15–26%), propionic (13–23%), iso-valeric (12–18%) and valeric (4–11%) acids, with little of iso-butyric (6–9%) and trace amounts of caproic (<1%) acids (Fig. 11). Lactic acid levels were below to 10 mg COD/L (Morgan-Sagastume *et al*. 2015). The VFA spectrum that was produced without pH control (5.5–6.5) appeared to regulate primarily by the sludge characteristics, such as carbohydrates, fat and protein compositions, which were thereby understood to be consistent among batches. WAS fermentation was conducted without pH control using the extant sludge buffering capacity, and thereby avoiding process costs associated with chemical addition. The levels of VFA obtained in their work by using a self-buffering fermentation without chemical addition for pH control are comparable and even higher levels than VFAs levels were obtained at laboratory scale with pH values 7-10 (Morgan-Sagastume *et al.* 2015).

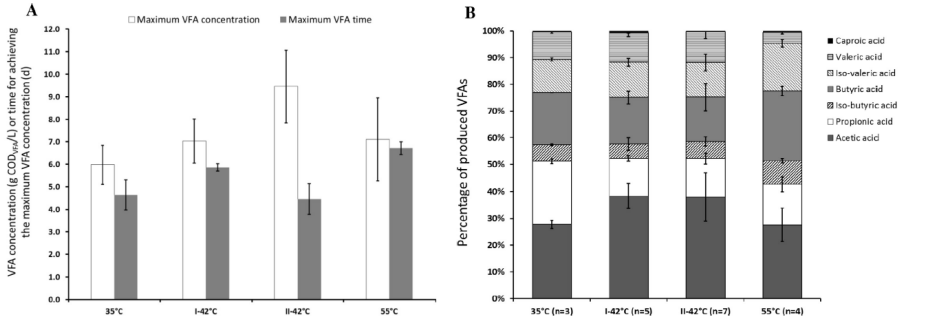

Figure 11. (A) Maximum VFA concentrations and respective retention times, and (B) COD-based percentage distribution of VFAs produced at maximum concentrations during the batch acidogenic fermentation of WAS at 35 ºC, 42 ºC and 55 ºC. Average ± standard deviations are reported for a population sample n. Values obtained during an initial batch testing (I-42 ºC) and a later routine fermentation (II-42 ºC; n = 5–11) are reported at 42 ºC. *(Image from Morgan-Sagastume et al. 2015).*

Furthermore, Tamis *et al.* (2014) carried out the production of PHA from a candy bar factory, so an industrial wastewater. The pilot plant consisted of two anaerobic reactors operated in series (see Appendix 5, Fig. 2). The temperature was maintained at 30 ± 0.1 ºC in first reactor, and 40 ± 0.1 ºC in the second reactor. Unlike Morgan-Sagastume *et al.* (2015), he and his coworkers maintained a pH of 4.5 ± 0.1 by addition of 1 M NaOH. The NaOH consumption for maintaining the pH of both reactors at 4.5 was on average 5 mol/day, equal to around 2 mmol/gCOD.

It is important to note that a significant net production of ethanol and VFAs was observed in the anaerobic upflow sludge bed reactor (the first step of the two-step fermentation). Subsequent fermentation in the second anaerobic fermentation reactor resulted in a net conversion of ethanol to VFAs (Fig. 12 (a)). During the fermentation stage, Bengtsson *et al.* (2008) obtained the results shown in Figure 12 (b). The outlet of the fermenter contained on average 74% VFA of the soluble COD as compared to 14% in the influent stream. Of the soluble COD consumed, 75% was converted to VFA, 18% was converted to biomass and the remaining (7%) was lost in the conversion, supposedly to gas production. Bengtsson *et al*. (2008) carried out the production of PHA by activated sludge treating a paper mill wastewater. He worked with two reactors (See Appendix 5, Fig. 6), and both of them were aerated and temperature controlled at 30 °C. Like Tamis *et al.* (2014) and Palmeiro-Sáncehz *et al*. (2014), Bengtsson *et al.* (2008) kept pH to 7.3 in the main reactor by addition of 2M HCl.

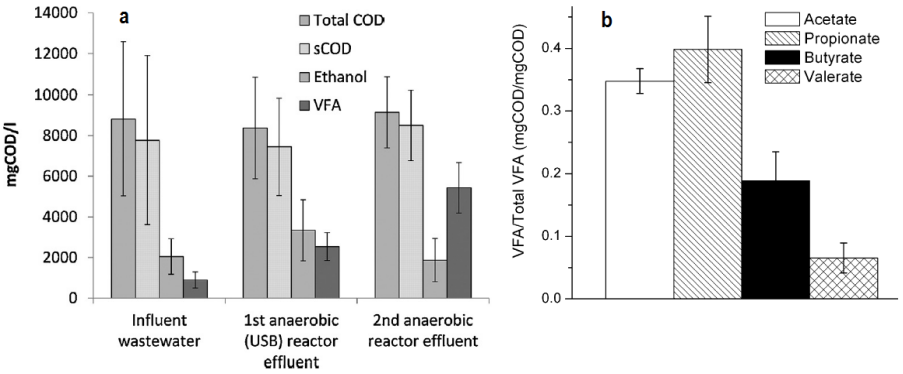

Figure 12. (a) Average concentrations of COD in the wastewater before and after fermenta-tion. The COD concentrations of the incoming wastewater varied substantially fromday to day and this was

represented by the error bars (±standard deviation over thedataset). (Image from J. Tamis et al. 2014). (b) Concentration of total VFA in the outlet of fermentor over the course of the experiment.

(Images from Tamis et al. 2014 and Bengtsson et al. 2008).

Tamis *et al.* (2014) accomplished an average fraction of VFA in the soluble COD of 0.64 ± 0.15 gCOD/gCOD after the two fermentations and the VFA compostition was acetate (32 ± 6%), propionate (14 ± 3%), butyrate (33 ± 11%), valerate (5 ± 3%), and caproate (18 ± 8%, COD basis). Finally, there is a need to mention the experiment carried out by Palmeiro-Sánchez *et al.* (2014). She and her coworkers used wastewater from washing of tuna kettle of a Galician company to produce PHA. The experimental configuration of the process is shown in Appendix 5, Fig.3. In this experiment, the temperature was maintained at 30ºC but there was no pH control. It also is important to indicate that the nitrification was avoided by addition of 1.5 mL L^{-1} of allylthiourea (ATU) (33 g L^{-1}), as the experiment carried out by Morgan-Sagastume *et al*. (2015). The results obtained in the composition of the VFA are shown in Table 11.

Table 11. Features composition of VFA obtained by different authors.

VFA [%]	Morgan-Sagastume *et al.* (2015)	Tamis *et al.* (2014)	Palmeiro-Sanches *et al.* (2014)	Bengtsson *et al.* (2008)
Acetic acid	28 – 38	32 ± 6	58	35
Butyric acid	15 – 26	33 ± 11	6.4	19
Propionic acid	13 – 23	14 ± 3	24	40
i-valeric acid	12 – 18	*	2.4	*
Valeric acid	4 – 11	5 ± 3	9.2	55
Caproic acid	<1	18 ± 8	*	*

(*) Information not available.

Accordind to Morgan-Sagastume *et al.* (2015), a similar dominance of acetic, butyric and propionic acids among VFA products was observed in the acidogenic fermentation of thermally pre-treated sludge, where the sludge characteristics rather than the fermenter's retention time determined the VFA product spectrum. For that reason, the sludge nature has been reported to influence VFA composition in fermentation (Pittmann and Steinmetz, 2013), and consistency in sludge characteristics would be a factor ultimately influencing consistency in copolymer composition for a PHA-production process within municipal treatment infrastructures.

7.2. ENRICHMENT REACTOR

In this stage, Morgan-Sagastume *et al.* (2015) produced enrichment biomass with the feast-famine treatment of RBCOD from municipal wastewater with daily and seasonal variations in wastewater characteristics and temperature (Fig. 10). In this case, the wastewater was been treated in order to eliminate quantities of COD, COD_{SOL}, N and P (70 ± 6, 60 ± 5, 24 ± 3 and 46 ± 7, respectively). Nevertheless, Tamis *et al.* (2014) produced enriched biomass by the stream from the second anaerobic reactor (Appendix 5, Fig.2). In a similar way, Palmeiro-Sáncehz *et al.* (2014) produced enriched biomass by the current of fermentation reactor (Appendix 5, Fig. 3). In another study conducted by Moralejo-Gárate *et al.* (2014), which consists in producing bioplastics from wastewater, PHA without taking into account the stage of acidogenic fermentation is produced in order to reduce the process costs (Appendix 5, Fig. 7). Therefore, this process has only two stages (enrichment stage and accumulation stage).

According to the results obtained by Morgan-Sagastume *et al.* (2015), the feast period lasted 10 -15 min and according to Tamis *et al.* (2014), the length of the feast phase varied initially between 30 and 90 minutes, but it became shorter and more stable (35 ± 5 min) after day 50 of operation. The end of this period activity was indicated by a DO increase. The minimum and maximum DO levels varied during the day due to the diurnal variations in influent strength. Famine prevailed during the following second aerated period after effluent discharge when the DO levels increased (Morgan-Sagastume *et al*. 2014).

Tamis and Palmerio-Sánchez (2014) obtained similar results regarding to the trend of DO levels during the period feast-famine. The difference in the operation method lies in which Tamis *et al.* (2014) conducted the experiment with a pH control, thus, the substrate was dosed intermittently to maintain a minimum pH of 6.5 due to its acidic nature. Shortly after dosing the substrate, a sharp increase in pH was observed concurrently with a sharp increase of the DO concentration, indicating that VFA was depleted (Fig. 13). The pH profile during the famine phase was influenced by various factors: shortly after the feast phase the pH had the tendency to increase due to stripping of CO_2 and hydrolysis of the urea in the dosed nutrients, and on average 1.8 mol/cycle HCl was dosed keep the pH below 7.5. Later in the famine phase, the pH decreased due to ammonium uptake and on average 0.2 mol/cycle NaOH was dosed to keep the pH above 6.5 (Tamis *et al.* 2014).

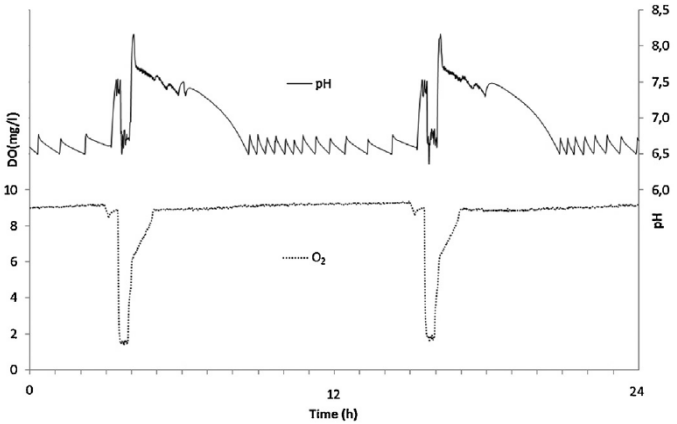

Figure 13. DO and pH profiles of the operational cycle in the enrichment reactor.

(Image from Tamis et al. 2014)

Another parameter used to assess the degree of enrichment of the culture is the consumption distribution of ammonium along cycle. As seen in Figure 14, ammonium consumption occurs, mainly, during the famine, when the substrate (the substrate in the experiment of Moralejo-Gárate *et al.* (2014) was glycerol) has been exhausted. This phenomenon indicates that the biomass growth in the reactor is due to enrichment polymers consumption stored during the satiety (Moralejo-Gárate *et al*. 2014).

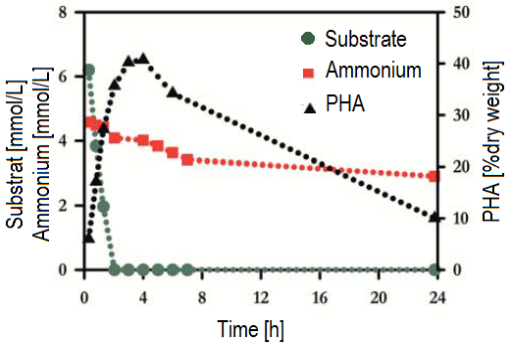

Figure 14. Evolution of the concentration of substrate (glycerol) and ammonium, and cell content PHA by dry weight of the reactor during a cycle of enrichment.

(Image from Helena Moralejo-Gárate *et al. 2014)*

The average sludge concentration that Tamis et al. (2014) obtained at the end of the cycle was 1.5 ± 0.4 gVSS/l while Morgan *et al.* (2015) got a biomass production of 250 gVSS/d.

Morgan got biomass yields of 0,35 - 0,45 gVSS / gCOD while the overall yield of biomass on COD that Tamis *et al.* (2014) obtained was 0.33 gVSS / gCOD. These differences in values may be due to the variations in the composition of the wastewater, and also, due to the difference in the process model carried out by each of them. The results obtained for each authors are shown in the following table.

Table 12. Results obtained in the reactor enrichment from both different experiments.

Parameter	Tamis *et al.* 2014	Morgan-Sagastume *et al.* 2015
Biomass production	1.5 ± 0.4 gVSS/L	250 gVSS/d
Biomass yield	0.33 gVSS/COD	0.35 – 0.45 gVSS/gCOD

It should be noted that the content of PHA increases during the feast phase when VFA are present. This fact can observe in Figure 15. At the end of the feast phase all VFA is consumed. The PHA content of biomass, which was achieved in the experiment of Tamis *et al*. (2014), was 0.40 ± 0.05 gPHA/gVSS. As it can be seen in Figure 15, there is an increase of VSS at the same time that increases the PHA content, indicating that the active biomass or other storage compound was produced simultaneously with the production of PHA. This same figure (Fig. 15), after the VFA is consumed, in the famine phase, shows that there is still soluble COD present in the reactor, mainly on the form of ethanol, but subsequent uptake of this soluble COD did not increase the amount of PHA in the system. Instead, PHA is degraded while the amount of VSS increases throughout the remainder of the cycle, indicating growth on storage.

As far as the influence of the substrate concentration, it can say that the substrate concentration affects the kinetics of substrate consumption and polymer storage. Polymer production rates and substrate uptake rates increase with an increasing substrate concentration up to a maxim value, then these values decrease again due to inhibition by substrate. Inhibition by substrate has been demonstrated in batch production by several authors. In continuous systems, the substrate concentration is constant and, therefore, a constant pressure for PHA-storing organisms is maintained to, while in SBRs, the substrate concentration is not constant, thus the substrate uptake rate and the pressure for the PHA-storing organisms can decline. Nevertheless, the SBR is more flexible than continuous system because the influent substrate concentration can be controlled by adjusting the length of the feed phase.

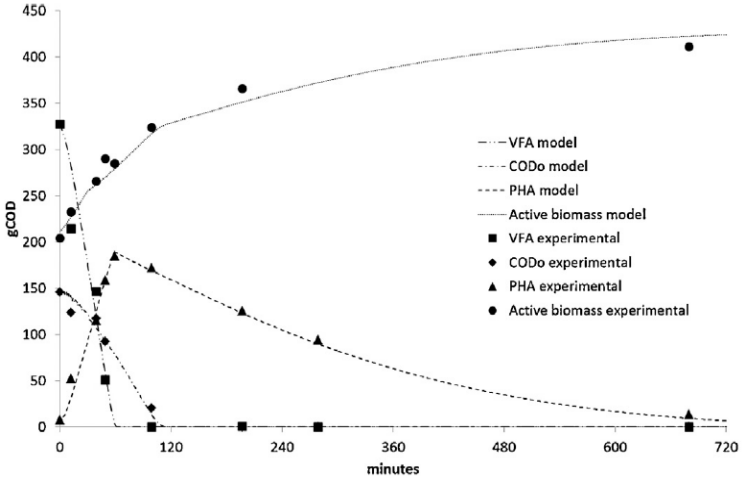

Figure 15. Detailed characterization of a representative cycle (day 58) including measurements of the substrate, PHA and active biomass amounts.

(Image from Tamis et al. 2014).

7.3. ACCUMULATION REACTOR

In the following scheme (Fig. 16) is shown the stage of accumulation process carried out by Tamis *et al.* (2014). This process is similar for all the other authors. The accumulation reactor was fed with fermented wastewater using a pH controlled pump during a period of 4 - 7 h. The outlet of enrichment reactor contained active biomass concentrations of 1.5 g/L and initial ammonium, nitrite and nitrate concentrations lower than 5 mg N/L.

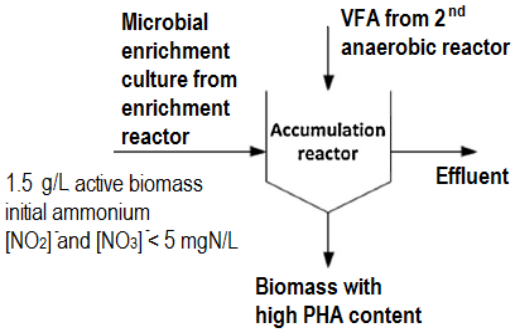

Figure 16. Scheme of stage of the accumulation reactor conducted by Tamis *et al*. (2014).

According to Tamis *et al*. (2014) the average PHA content after accumulation was 0.70 ± 0.05 gPHA/gVSS and a maximum PHA content achieved was 0.76 gPHA/gVSS. It was not possible to identify the PHA yields of the different substrates present in the fermented wastewater independently.

Figure 17 shows the evolution of PHA content and the consumption of substrate in the accumulation reactor (Moralejo-Gárate *et al.* 2014). During the first five hours the culture was able to accumulate up to 57% PHA biomass dry weight, reaching 70% after 24 hours.

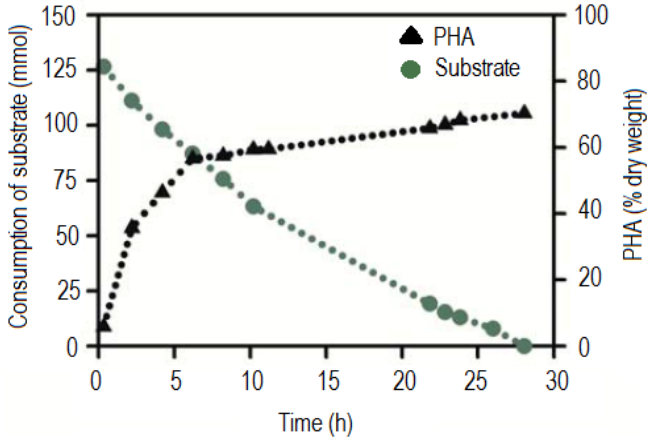

Figure 17. Evolution of substrate consumption and cell content of PHA in dry weight.

(Image from Helena Moralejo-Gárate *et al. 2014)*

In Bengtsson *et al.* (2008) study, the nutrient levels were varied in the parallel batch experiments to provide an indication of sensitivity of the storage and growth response during accumulation (Fig. 18). He obtained a significant PHA accumulation, but with some differences between the experiments. With nutrient limitation, 43–48% PHA of TSS was achieved, while the excess nutrients resulted in much lower PHA content (32% as maximum). Furthermore, a distinct biomass growth response after 10 h of accumulation was produced (Fig. 19). The amount of biomass produced was much less in the cases with N and/or P limitation. Thus, in these cases where there was a nutrient limitation, PHA yields were higher than for nutrient excess.

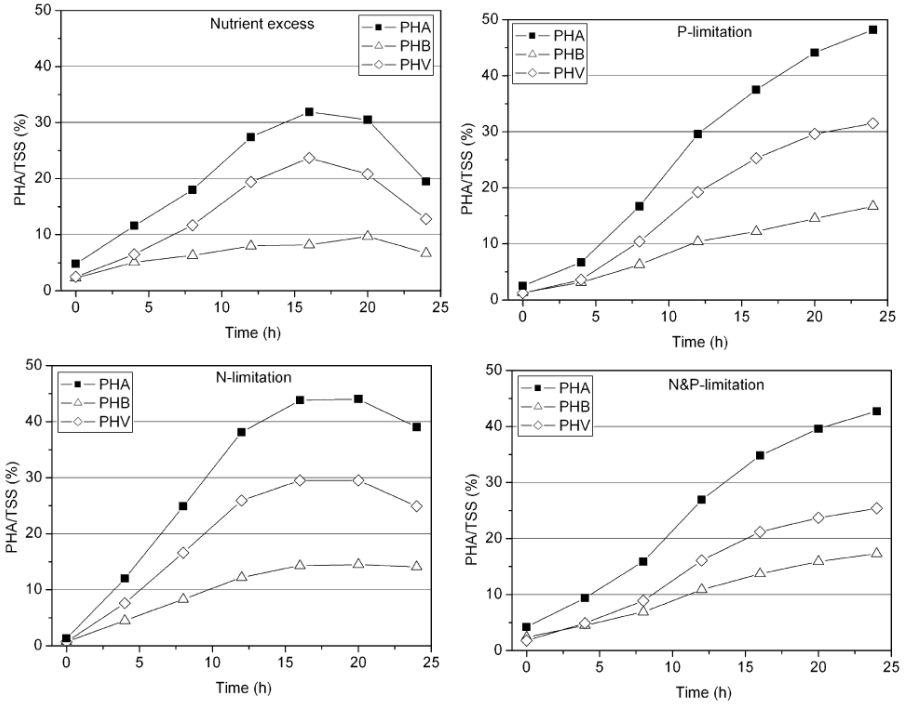

Figure 18. PHA content in the sludge during batch experiments with different nutrient concentrations.

(Image from Bengtsson et al. *2008).*

The measured PHA content of the sludge was expressed as:

$$PHA\ \% = \frac{PHB+PHV}{TSS} 100\%\ (g/g) \quad (1)$$

TSS was assumed to be composed of active biomass (X) and PHA (Jiang *et al.* 2011).

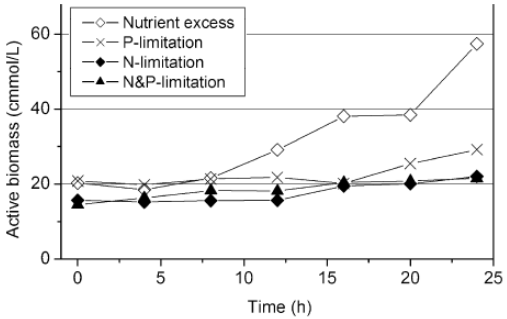

Figure 19. Concentration profiles of active biomass during the batch experiments.

(Image from Bengtsson et al. 2008).

It has shown that nutrient limiting conditions lead to higher PHA content and higher PHA yields (Bengtsson *et al* 2008), because during this nutrient limitation, the cells are unable to multiply and remain almost constant. Nonetheless, the cells begin to increase in size and weight owing to intracellular accumulation of PHA as a storage product. Nutrient excess resulted in higher biomass growth rate compared to all the cases with nutrient limitation which suggests that cell growth was limited in absence of nutrients (Table 13).

Table 13. Biomass growth rate obtained in different conditions. *(Bengtsson et al. 2008).*

Parameter	Nutrient excess	P-limitation	N-limitation	N and P-limitation
PHA [%TSS]	31.9	48.2	44.0	42.7
μ	0.31	0.18	0.13	0.26

It has also been observed that the use of synthetic substrates enables higher levels of enrichment with regards to PHA producing bacteria (0.70 *vs.* 0.90 gPHA/gVSS, Tamis *et al.* 2014). With the aim of explain these differences between the maximums PHA contents obtained, according to Tamis *et al.* (2014) we have take into account three factors: (1) presence of solids in the fermented wastewater, (2) the presence of a non-storing side population, and (3) the production of other types of storage compounds from ethanol.

In Table 14 shows the yield of the process according to the values of PHA obtained by different strategies.

Table 14. PHA content obtained by different authors.

$Y_{PHA/COD}$	Reference
0,33	Morgan-Sagastume et al. 2015
0,30	Tamis et al. 2014
0,13	Bengtsson et al. 2008
0,22	Valentino et al. 2014
0,65	Albuquerque et al. 2008
0,33	**Average value**

8. ECONOMIC POTENTIAL FOR PHAS PRODUCTION FROM MUNICIPAL WASTE

Municipal wastes have a significant fraction of organic matter. An alternative to the production of biogas during the treatment of this waste would be the PHA production.

This section consists in to make a preliminary estimate of the amount of PHA that can be obtained from municipal wastes to determine whether the process is profitable or not. On the one hand, based on inputs in an ECOPARC, an estimate of the amount of PHA that can be obtained and the benefits and costs involved in this production is calculated. On the other hand, a rough estimate of equipment costs for the industrial production of PHA has been done (Table 15 and 16).

Researchers have reported that the price of PHAs produced by current and potential large scale manufacturers is in the range of €1.5 – 5 per kg PHA. A value of 3€/kg PHA is assumed to be a currently competitive price for a ''green'' polymer product (Gurieff and Lant, 2007).

The following calculations are made based on annual production of 2.503 tones of PHA from municipal waste. According to the ECOPARC of Barcelona (*www.ecoparcbcn.com*) annually receives 228231.15 tones of municipal waste, which are 74552.45 tones of OFMW. From this last amount, and given the following data for the OFMW:

TS = 40% of wet weight $\longrightarrow \frac{40\ tone\ TS}{100\ tone\ OFMW} \cdot 100\%$

VS = 70 – 95% of TS $\longrightarrow \frac{(70-95) tones\ VS}{100\ tones\ TS} \cdot 100\%$

1g VS = 1.49g COD $\longrightarrow \frac{1.49\ tones\ COD}{1\ tone\ VS} \cdot 100\%$

VFA = 20% of COD $\longrightarrow \frac{20\ tones\ VFA}{100\ tones\ COD} \cdot 100\%$

A production of PHA of around 2500 tones annually is obtained. As shown in Table 14, the yield of the process is about 33%. The analysis of PHA production can be calculated as follows:

$$74\,552.45\ \frac{t\ OFMW}{year} \cdot \frac{40\ t\ TS}{100\ t\ OFMW} \cdot \frac{70\ VS}{100\ t\ TS} \cdot \frac{1.49\ t\ COD}{1\ t\ VS} \cdot \frac{20\ t\ VFA-COD}{100\ t\ COD} \cdot \frac{33\ PHA}{100\ VFA-COD} = 2.503\ {tones\ PHA}/{year}$$

Therefore, if during a year it can produce up to 2.503 tones of PHA from municipal waste and assuming that 1 kg of PHA sells for 3€, it can get the income which is calculated next:

$$2.503 \frac{t\ PHA}{year} \cdot \frac{1000\ kg\ PHA}{1\ t\ PHA} \cdot \frac{3\ €}{1\ kg\ PHA} = 6.158.449\ {}^{€}/_{year}$$

Finally, assuming that to produce 1 kg of PHA requires 6 kWh used for electricity and 3,75 kWh used for the steam (Tamis *et al.* 2014), given that 1 kWh costs 0,13€, operating costs are:

$$2.503\ \frac{t\ PHA}{year} \cdot \frac{1000\ kg\ PHA}{1\ t\ PHA} \cdot \frac{6\ kW\ h}{1\ kg\ PHA} \cdot \frac{0.13\ €}{1\ kW\ h} = 1.952.340\ {}^{€}/_{year}$$

It would cost 1.952.340 €/year approximately for the electricity costs.

$$2503\ \frac{t\ PHA}{year} \cdot \frac{1000\ kg\ PHA}{1\ t\ PHA} \cdot \frac{3.57\ kW\ h}{1\ kg\ PHA} \cdot \frac{0.13\ €}{1\ kW\ h} = 1.161.642{,}3\ {}^{€}/_{year}$$

And, for the steam costs, it would cost 1.161.642,3 €/year. Therefore, the economic potential that suppose a production of 2500 tones of PHA is calculated of the following way:

$$\text{Economic potential} = \text{Income} - \text{Costs of operation} \quad (2)$$

EP = 6.158.449 € - (1.952.340 + 1.161.642,3) € = 3.144.466,7 €/year

The purchase cost of each item of equipment has been estimated using literature correlations of the form (Van Wegen *et al.* 1998):

$$Cost = 10^{\alpha} \cdot Z^{\beta} \quad (3)$$

Where Z is the unit capacity used for cost estimated.

As a basis for carrying out these calculations, the flowchart shown in Figure 10 is taken. Fermenters have been assumed to be stirred tanks made of 304 stainless steel, where the cost of controllers and other associated fittings are included in the Lang factor (Van Wegen *et al*. 1998). Furthermore, the equipments which are shown in Figure 10 are taken to carrying out these calculations. It has been made a direct proportionality in order to do a sizing with a similar

plant used in the present work. The value of direct proportion equal to 0,58, *i.e.*, the values of Z which used in this section are 58% of the Z values in the similar plant (see Appendix 7). The total fixed capital cost and required working capital are estimated from the total equipment purchase cost, using the Lang Factors for a fluid processing plant (Table 16).

Table 15. Cost of main equipment for an industrial production of PHA using Lang Factors. *(Van Wegen et al. 1998 and Gurieff et al. 2007)*

Unit	Cost relations		Capacity correlation to be calculated	Estimated value for Z	Cost = $10^{\alpha} \cdot Z^{\beta}$	
	α	β			Cost [$]	Cost [€]
F	3,96	0,54	V [kL]	170	146030,11	131742,96
SBR	3,96	0,54	V [kL]	170	146030,11	131742,96
A	3,96	0,54	V [kL]	100	109647,82	98920,21
C 1	3,69	0,33	Effective area [m^2]	84800	207190,88	186919,94
C 2	3,69	0,33	Effective area [m^2]	84800	207190,88	186919,94
U	3,65	0,56	V [kL]	325	113934,35	102787,36
D	4,58	0,37	Drum area [m^2]	10	89125,09	80405,36
DAF	2,85	0,94	Brake power [kW]	730	347955,33	313912,42
Df	3,69	0,33	Inlet flow rate [m^3/h]	37	16125,25	14547,61
				TOTAL COST	1383229,82	1247898,77

As shown in Table 15, the estimated capital required for the production of PHA according to Lang Factors is about 1.247.898,77€. About this cost, it is necessary to add the cost of installation, start up expenses, working capital, etc. Therefore, the total estimated cost required is 7.637.140,46€ approximately as shown in Table 16.

Table 16. Fixed capital estimation using Lang Factors. *(Van Wegen, Ling and Middelberg, 1998)*

Equipment purchase cost:	100	%
Installation, ancillary equipment, site upgrade, etc**:**	246	%
Total direct cost:	346	%
Indirect costs, contractors fee, contingency:	137	%
Total fixed capital investment:	483	%
Working capital:	86	%
Startup expenses:	43	%
Total capital investment:	**612**	**%**
Total capital investment:	**7.637.140,46**	**€**

In the other hand, the summary of market prices for PHAs in 2010/2003 is given in the next table.

Table 17. *Major companies producers of PHA and selling price of the product (Chanprateep et al. 2010)*

Carbon source	Price per kg [€]	Yield of PHA [g/g]	Cost of C-source per kg of PHA	Company	Product	Product price [€/kg]	Production [t/year]
Sucrose	0.35	0.40	€ 0.87	Biotechnology Co., Germany	Biomer	20 (2003) 3-5 (2010)	50 (2003)
Glucose	0.41	0.38	€ 1.07	ICI, Engalnd	Biopol	-	300 (2010)
Ethanol	0.31	0.50	€ 0.63	-	-	-	-
Methanol	0.28	0.43	€ 0.58	Mitsubishi GAS Chemical, Japan	Biogreen	2.5-3 (2010)	10000
Cassava starch	0.19	0.20	€ 0.94	-	-	-	-
Cane molasses	0.10	0.42	€ 0.24	PHB Industrial S.A., Brazil	Biocycle	10-12 (2003) 2.5-3 (2010)	1400 (2003) 30000-60000 (2010)
Palm oil	0.79	0.65	€ 1.22	-	-	-	-
Soya oil	0.92	0.70	€ 1.31	-	-	-	-

In this work, the price of the carbon source is null because waste and wastewater are used such as a raw material.

If in one year the plant can produce a profit of 3.144.466,7 €, but the cost of the equipment factory is about 7.637.140,46 €, it would take two years and a half approximately to recover the initial investment cost, and during these years, there not would be gains. Furthermore, in this estimated analysis of the potential economic, the manpower costs and maintenance costs have not been taken into account. Thus, the economic potential would be lower than calculated. So, it is necessary reduce the costs of the process in order to do the profitable process. In addition, should take into account the cost of extraction and purification of PHA, which increase the overall cost of the process significantly.

9. CONCLUSIONS

The present work has concluded that:

- The bibliographic results shown that the PHAs can have a promising future to replace the conventional plastics because of their desirable properties and its biodegradability.
- According to literature, it has also shown that the production of functional PHA-storing biomass from the treatment of municipal wastewater RBCOD and VFA sourcing from sludge fermentation indicate an opportunity for integrating PHA production with municipal wastewater. It can be concluded that the production process consists of three stages: (1) acidogenic fermentation to produce VFA, (2) enrichment of biomass under conditions of feast-famine regime and, (3) an accumulation process to produce a PHA-rich biomass. Studies have shown the feast-famine regime is specifically effective for selecting PHA producing bacterial.
- It has been seen that the production of PHA from municipal waste is a potential issue. The raw material cost of this process is zero, but the municipal waste need a treatment, therefore, the zero cost of the municipal waste can offset the cost of treatment. In any case, the PHA production process is a procedure that could become profitable through a more careful study. A part from the production and

waste treatment costs, it should consider the costs of the PHA recovery (extraction and purification cost), which tend to increase the price of the overall process. At this stage of the process, there would be three possible different situations, one of them would make the recovery process in the own production plant, which would mean a significant increase in the overall cost of the process; another option would be to make the recovery process in a centralized plant, which would reduce costs a lot; and as a last resort, the PHA-rich biomass could be sold, which it would suppose a source of income for the plant.

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11. ACRONYMS

A	PHA Accumulation
ADF	Aerobic dynamic feeding
ATU	Allylthiourea
AD	Anaerobic digestion
COD	Chemical oxygen demand
CODs	Chemical oxygen demand soluble
C1, C2	Centrifuge
DAF	Diffused air flotation
Df	Drum filter
DO	Dissolved oxygen
DQO	Demanda química d'oxigen
EP	Economic potential
F	Fermenter
FHS	Fermented hydrolyzed sludge
FF	Feast and famine
HRT	Hydraulic retention time
MCL	Medium chain length
MMC	Microbial mixed culture
M_n	Molecular mass
M_w/M_n	Polydispersity
OFMW	Organic fraction of municipal wastes
OLR	Organic loading rate
PAP	PHA accumulation potential
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PHHx	Polyhydroxyhexanoate
PP	Polypropylene
RBCOD	Readily biodegradable COD
SBR	Sequencing batch reactor

SCL	Short chain length
SRT	Sludge retention time
TSS	Total suspended solids
TS	Total solids
T_g	Glass transition temperature
T_m	Melting temperature
U	Unstirred tanks
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WWTP	Wastewater treatment plant
w	Weight
X	Active biomass
μ	Biomass growth rate

APPENDICES

APPENDIX 1: RESULTS OF BIODEGRADATION PROCESS

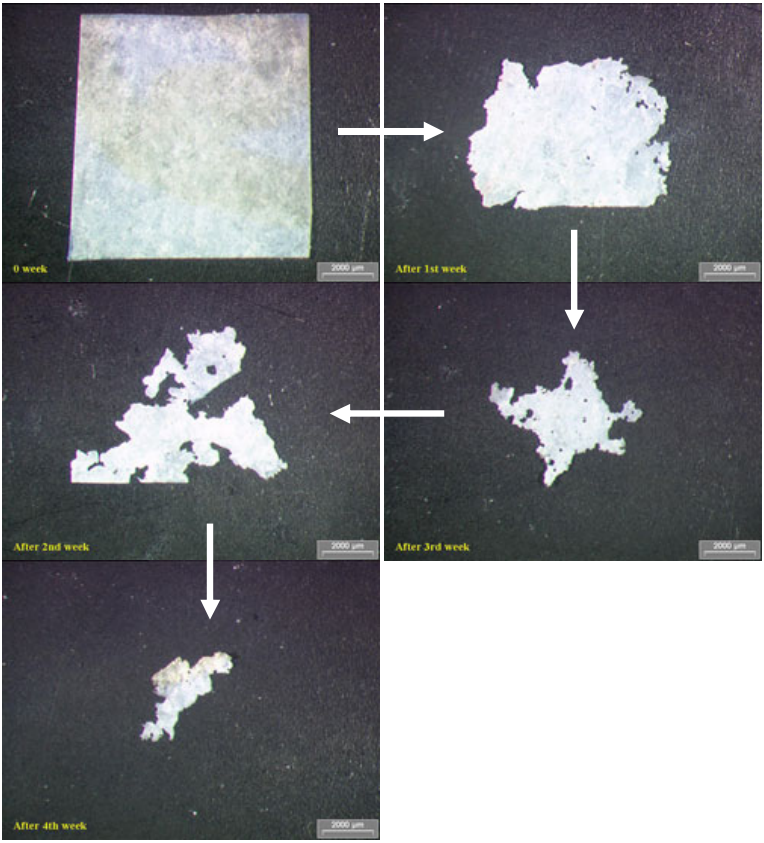


Image from Ecobiomaterial Web page, 20/04/2015.

APPENDIX 2: WORLDWIDE PHA PRODUCTION AND RESEARCH COMPANIES

Company	Type of PHA	Production scale [t/year]	Period	Applications
ICI, UK	PHBV	300	1980s to 1990s	Packaging
Chemie Linz, Austria	PHB	20-100	1980s	Packaging and drug delivery
BTF, Austria	PHB	20-100	1990s	Packaging and drug delivery
Biomers, Germany	PHB	Unknown	1990s to present	Packaging and drug delivery
BASF, Germany	PHB, PHBV	Pilot scale	1980s to 2005	Blending with Ecoflex
Metabolix, USA	Several PHAS	Unknown	1980s to present	Packaging
Tepha, USA	Several PHAS	Unknown	1990s to present	Medical bioimplants
ADM, USA (with Metabolix)	Several PHAS	50 000	2005 to present	Raw materials
P&G, USA	Several PHAS	Contract	1980s to 2005	Manufacture
Monsonto, USA	PHB, PHBV	Unknown	1990s	Raw materials
Meredian, USA	Several PHAS	10 000	2007	Raw materials
Kaneka, Japan (with P&G)	Several PHAS	Unknown	1990s to present	Packaging
Mitsubishi, Japan	PHB	10	1990s	Packaging
Biocycles, Brazil	PHB	100	1990s to present	Raw materials

Bio-On, Italy	PHA	10 00	2008 to present	Raw materials
Zhejiang Tian An, China	PHBV	2000	1990s to present	Raw materials
Jiangmen Biotech Ctr., China	PHBHHx	Unknown	1990s	Raw materials
Ecoman, Shandong, China	PHA	3000	2008 to present	Raw materials
Tianjin Northern Food, China	PHB	Pilot scale	1990s	Raw materials
Shantou Lianyi Biotech, China	Several PHAS	Pilot scale	1990s to 2005	Packaging and medicals
Jiangsu Nan Tian, China	PHB	Pilot scale	1990s to present	Raw materials
Shenzhen O'Bioer, China	Several PHAS	Unknown	2004 to present	Unknown
Tianjin Green Bio-Science	P3HB4HB	10 000	2004 to present	Raw materials and packaging
Shandong Lukang, China	Several PHAS	Pilot scale	2005 to present	Raw materials and medicals

(a) P3HBV = Copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate; (b) P3HBcoH3Hx = Copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate; (c) P3HBco4HB = Copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate.

(Information from Chen et al. 2009)

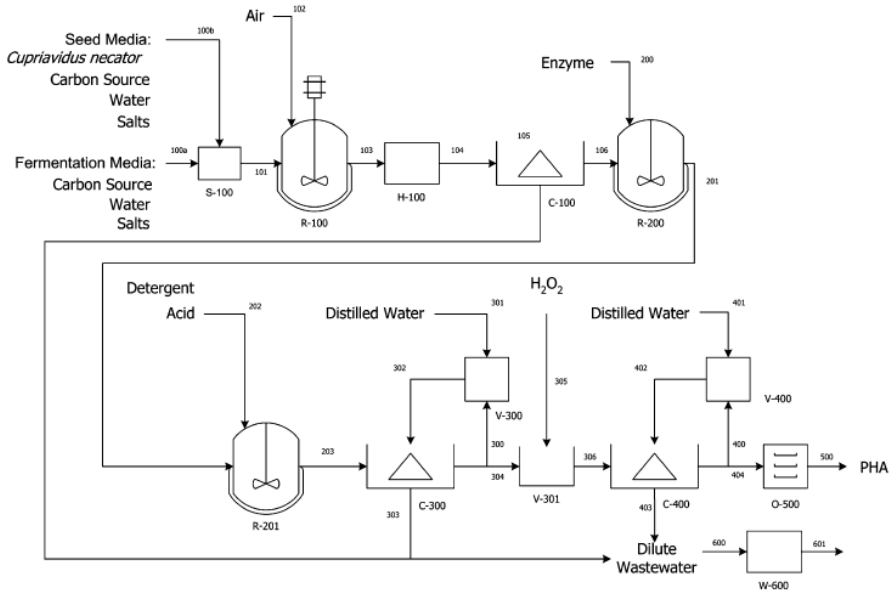
APPENDIX 3: BACTERIAL STRAINS USED TO PRODUCE PHA

Bacterial Strain (s)	Carbon source (s)	Polymer (s) produced
Aeromonas hydrophila	Lauric acid, oleic acid	mcl-PHAs
Alcaligenes latus	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB
Bacillus cereus	Glucose, caprolactone, sugarbeet molasses	PHB, terpolymer
Bacillus spp.	Nutrient broth, glucose, alkanoates, ecaprolactone, soy molasses	PHB, PHBV, copolymers
Burkholderia sacchari sp. nov.	Adonitol, arabinose, arabitol, cellobiose, fructose, fucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose, xylitol	PHB, PHBV
Burkholderia cepacia	Palm olein, palm stearin, crude palm oil, palm kernel oil, oleic acid, xylose, levulinic acid, sugarbeet molasses	PHB, PHBV
Caulobacter crescentus	Caulobacter medium, glucose	PHB
Escheruchia coli mutants	Glucose, glycerol, palm oil, ethanol, sucrose, molasses	(UHMW)PHB
Halomonas boliviensis	Starch hydolysate, maltose, maltotetraose and maltohexaose	PHB
Legionella pneumophila	Nutrient broth	PHB
Methylcoystis sp.	Methane	PHB
Microlunatus phosphovorus	Glucose, acetate	PHB
Pseudomonas aeruginosa	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	mcl-PHAs
Pseudomonas putida	Glucose, octanoic acid, undecenoic acid	mcl-PHAs
Pseudomonas stutzeri	Glucose, soybean oil, alcohols, alkanoates	mcl-PHAs
Rhodopseudomonas palustris	Acetate, malate, fumarate, succinate, propionate, malonate, gluconate, butyrate, glycerol, citrate	PHB, PHBV
Staphylococcus epidermidis	Malt, soy waste, milk waste, vinegar waste, sesame oil	PHB
Cupriavidus necátor	Glucose, sucrose, fructose, valerate, octanoate, lactic acid, soybean oil	PHB, copolymers
Cupriavidus necátor H16	Hydrogen, carbon dioxide	PHB

APPENDIX 4: INPUT STREAM CHARACTERISTICS IN THE FERMENTATION REACTOR

Ref.	Tamis et al. 2014	Moralejo -Gárate et al. 2014	Bengtsson et al. 2008	Morgan-Sagestume et al. 2015	Jiang et al. 2011	Johnson et al. 2009	Karlsson et al. 2010	Moralejo -Gárate et al. 2013	Moralej o-Gárate et al. 2013	Morgan et al. 2011
FEED MEDIUM										
Source carbon	Ethanol	154 mM $C_3H_6O_3$	870 mg/L acetate	Acetate or acetate/propionate	131 mM (NaPr)	270 mM $NaAc·3H_2O$	FHS	154 Mm $C_3H_6O_3$	154 mM $C_3H_6O_3$	16.2 mM Lactate
NUTRIENT SOURCE										
P	0.3M	-	-	38±8 mg/gVS	-	-	0.64 g/L	-	-	-
$MgSO_4$	0.3M	1.37 g/L	-	-	5.6 mM	5.55 mM	0.42 g/L	1.37 g/L	1.37 g/L	0.55 mM
K_2SO_4	0.2M	-	-	-	-	-	-	-	-	-
$FeCl_3$	64 mM	-	-	-	-	-	-	-	-	-
$ZnSO_4$	3 ml	-	-	-	-	-	-	-	-	-
H_3BO_3	2.7 mM	-	-	-	-	-	-	-	-	-
$NiCl_2$	2.1mM	-	-	-	-	-	-	-	-	-
$CoSO_4$	1.5 mM	-	-	-	-	-	-	-	-	-
$CuSO_4$	0.6 mM	-	-	-	-	-	-	-	-	-
Na_2MoO_4	0.8 mM	-	-	-	-	-	-	-	-	-
N	3M	-	-	80±11 mg/gVS	-	-	-	-	-	-
NH_4Cl	-	3.61g/L	1.24 g/L	-	67.5 mM	67.5 mM	2.5 g/L	3.61 g/L	3.61 g/L	6.74 mM
KH_2PO_4	-	3.39g/L	0.34 g/L	-	24.9 mM	24.9 mM	-	3.39 g/L	3.39 g/L	2.49 mM

APPENDIX 5: SCHEMES AND FLOW DIAGRAMS FOR PRODUCING PHAS FROM WASTE AND WASTEWATER



S-100 – Steam sterilisation
R-100 – Microbial Growth Reactor
H-100 – High Pressure Homogeniser
C-100 – Centrifugation
R-200 – Enzyme Washing Reactor
R-201 – Detergent Washing Reactor
C-300 – Centrigugation
V-300 – PHB Re-suspension
V-301 – Hydroxide Addition
C-400 – Centrifugation
V-400 – PHB Re-suspension
O -500 – Spray Drying
W-600 – Wastewater treatment

Figure 1. *Process flowsheet for PHB production*
(Image from Harding et al. (2007). Journal of Biotechnology 130. 57–66)

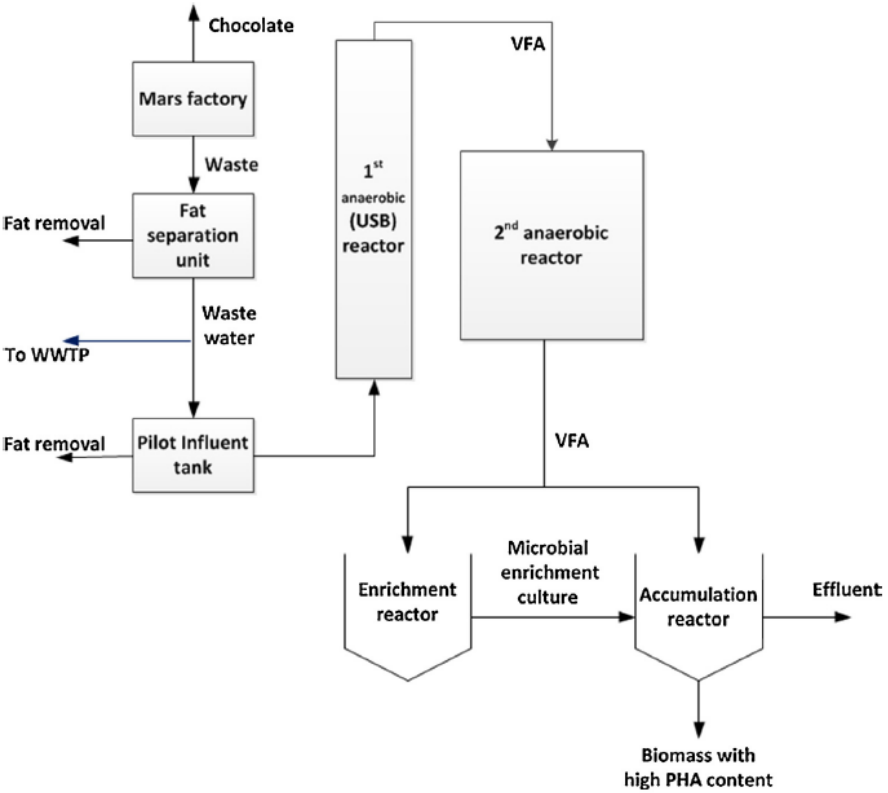


Figure 2. *Schematic overview of the pilot system for PHA production from wastewater at the Mars candy bar factory, Veghel, The Netherlands.*
(Image from Tamis et al. (2014). Journal of Biotechnology 192. 161–169).

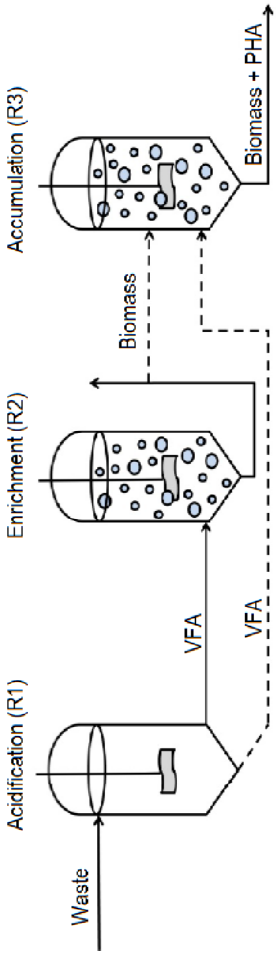


Figure 3. *Experimental configuration biopolymer production process in three steps: acidification of the residue, culture enrichment and maximum accumulation of biopolymer, respectively. (Image from Palmeiro-Sánchez et al. (2014)).*

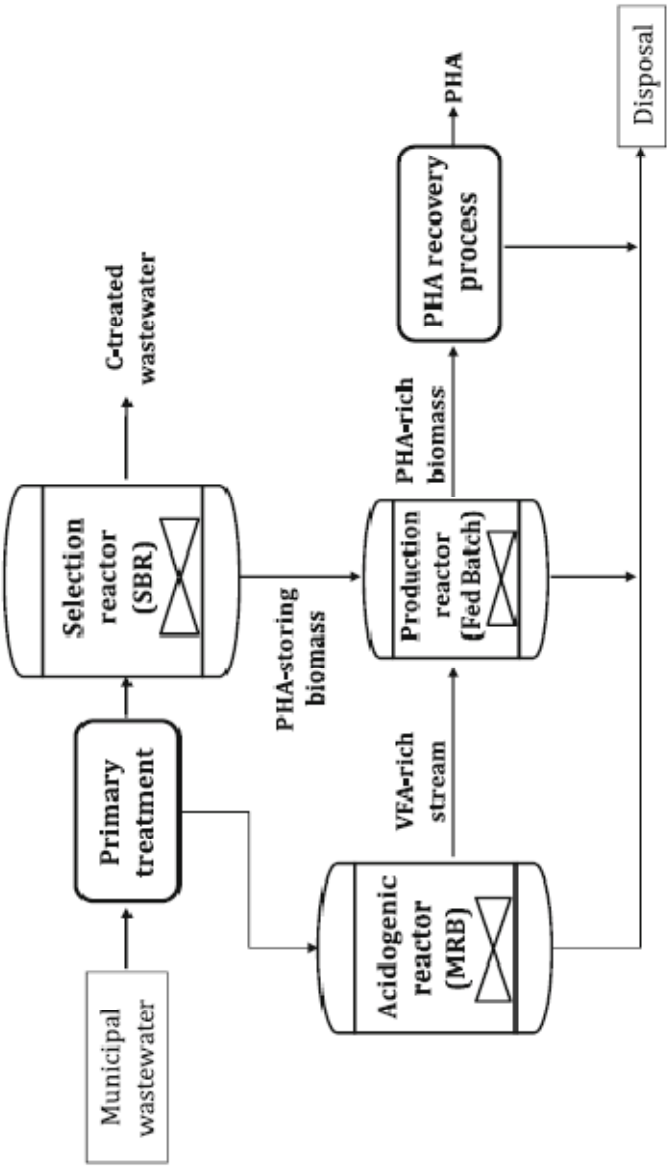


Figure 4. *Schematic process flow diagram of municipal wastewater and sludge treatment in conjunction with PHA production under study in the project ROUTES. (Image from Valentino et al. (2014)).*

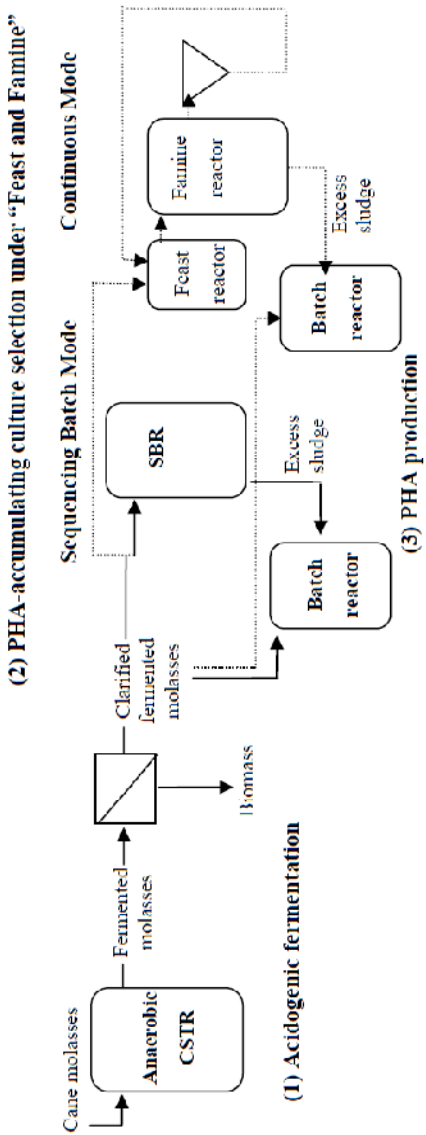


Figure 5. *3-Stage pha production process from sugar molasses by mixed cultures using either a Sequencing Batch Reactor (full line) or a continuous ADF system (dashed line) to carry out culture selection.*
(Image from Albuquerque et al. (2008)).

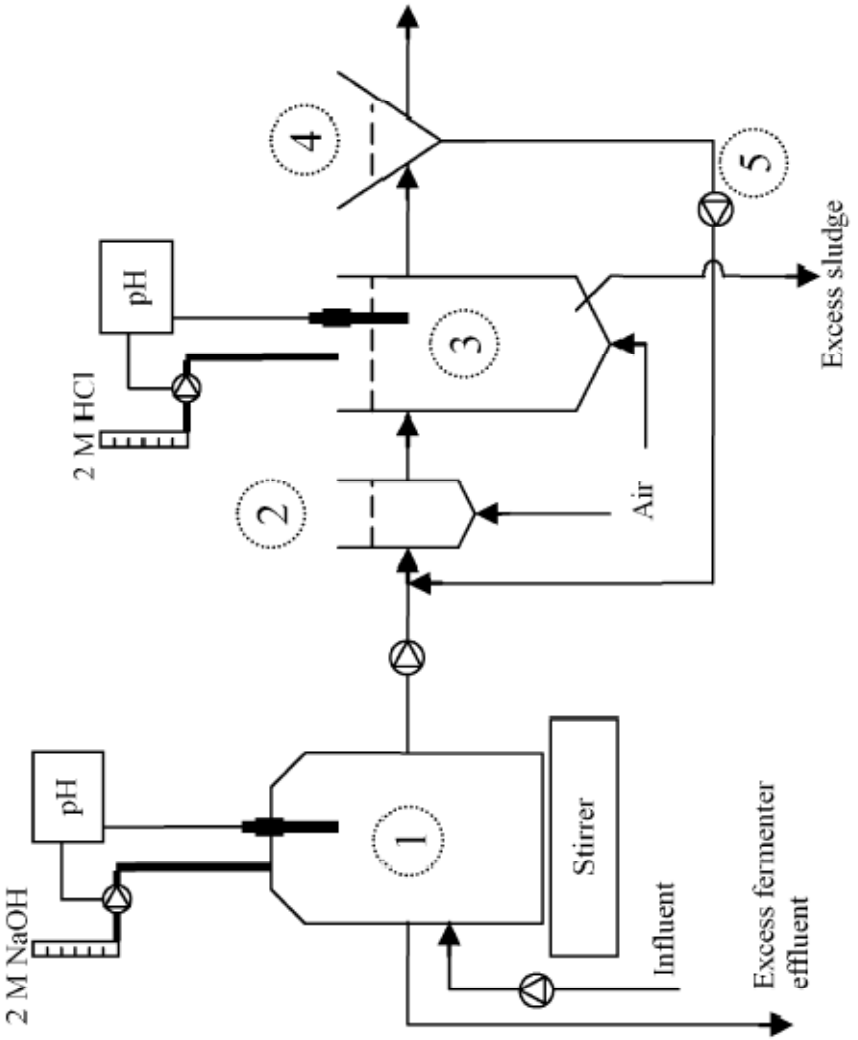


Figure 6. *Reactor set-up for continus experiment with (1) acidogenic fermentation, (2) selector, (3) main reactor, (4) clarifier, (5)sludge return pump. (Image from Bengtsson et al. (2008)).*

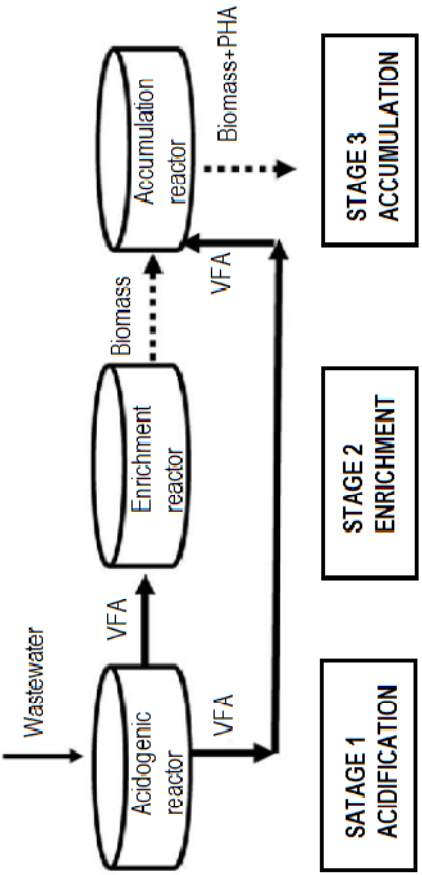


Figure 7. *Scheme of PHA production process in mixed culture using wastewater as fermentable substrate.*
(Image from Moralejo-Gárate et al. 2014)

APPENDIX 6: FERMENTATION PERFORMANCE PARAMETERS BASED ON DIFFERENT BATCH SAMPLES

Parameter	Units	35 °C ($n = 3$)	I-42 °C ($n = 4$)	II-42 °C	55 °C ($n = 4$)
(B)					
Solubilization	$gCOD_{sol}/gCOD_{total\ init}$	0.15 ± 0.01	0.21 ± 0.04	0.25 ± 0.03 ($n = 8$)	0.22 ± 0.03
	$gCOD_{sol}/gVS_{initial}$	0.26 ± 0.02	0.29 ± 0.02	0.40 ± 0.03 ($n = 10$)	0.38 ± 0.02
VS reduction	$gVS/gVS_{initial}$	0.11 ± 0.04	0.18 ± 0.09	0.04 ± 0.05 ($n = 5$)	0.14 ± 0.01
TS reduction	$gTS/gTS_{initial}$	0.10 ± 0.05	0.19 ± 0.08	0.05 ± 0.04 ($n = 6$)	0.13 ± 0.01
VFA conversion	$gCOD_{VFA}/gCOD_{sol}$	0.81 ± 0.06	0.90 ± 0.10	0.85 ± 0.08 ($n = 7$)	0.70 ± 0.04
VFA yield	$gCOD_{VFA}/gVS_{initial}$	0.21 ± 0.01	0.27 ± 0.03	0.33 ± 0.03 ($n = 7$)	0.27 ± 0.02
	$gCOD_{VFA}/gCOD_{total}$	0.13 ± 0.01	0.20 ± 0.04	0.22 ± 0.03 ($n = 7$)	0.17 ± 0.02

n: batch samples

(Image from Morgan-Sagastume et al. 2015)

APPENDIX 7: RESULTS OF ECONOMIC ANALYSIS

Unit	#	Total Size	Total Cost
Inoculator F203	1	14,4 kl	$46k
Fermenters F201, F202	2	578 kl	$461k
Homogenizer H301	1	65,0 $m^3 h^{-1}$	$430k
Centrifuges C401	8	1.170.000 m^2	$1.983k
Compressors A201	2	2.510 kW	$1.545k
Dryers D501	3	46,0 m^2	$385k
Extruder & Pelletizer E501	1	540 kg h^{-1}	$126k
Sterilizer S101	1	14,4 m^3h^{-1}	$463k
Tank T101	1	260 m^3h^{-1}	$101k
Tanks T301, T302, T401	3	867 kl	$322k
Tank T402	1	215 kl	$91k
Tank T501	1	84,0 kl	$54k

(Table from Van Wegen et al. 1998)

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