



Universidade de
Aveiro

Departamento de Química

2013

**Ana Lúcia
Morgado Ferreira**

**Isolamento e caracterização de bactérias
acumuladoras de PHAs em HSSL**

**Isolation and characterization of PHAs-accumulating
bacteria from HSSL**



Universidade de
Aveiro

Departamento de Química

2013

**Ana Lúcia
Morgado Ferreira**

**Isolamento e caracterização de bactérias
acumuladoras de PHAs em HSSL**

**Isolation and characterization of PHAs-accumulating
bacteria from HSSL**

Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, branch in Industrial and Environmental Biotechnology, performed under the scientific guidance of Prof. Luísa Seuanes Serafim, Invited Assistant Professor at Department of Chemistry, University of Aveiro, and Dr. Simona Rossetti, Researcher at Istituto di Ricerca sulle Acque – Consiglio Nazionale delle Ricerche (IRSA/CNR).

Dedico este trabalho aos meus pais, Jorge e Lúcia, aos meus irmãos, Bruno e Clara, e ao André.

the jury

president

Prof. Ana Maria Rebelo Barreto Xavier
Assistant Professor at Department of Chemistry, University of Aveiro

Prof. Luísa Alexandra Seuanes Serafim Leal
Assistant Professor at Department of Chemistry, University of Aveiro

Dr. Simona Rossetti
Researcher at Istituto di Ricerca sulle Acque – Consiglio Nazionale delle Ricerche

Dr. Nuno Filipe Ribeiro Pinto de Oliveira Azevedo
Assistant Researcher at Department of Chemical Engineering/Faculty of Engineering, Nova University of Porto

acknowledgments

I would like to thank to Prof. Luísa Serafim and Dr. Simona Rossetti for their patience, help throughout the year and for the wonderful opportunity.

To people at LEB and IRSA for their welcoming and friendship, especially to Diogo and Cristina, my two un-official supervisors, for all their help and patience.

I also wish to thank my closest friends for their constant support and presence beyond borders.

To André for the unconditional patience, help and love.

Finally, a special thanks to my parents, my brother and sister, for their belief in me, for all the opportunities they gave me and all their love and support.

palavras-chave

Polihidroxicanoatos, Culturas Mistas Microbianas, HSSL, FISH, PCR, 16S rDNA

resumo

Polihidroxicanoatos (PHAs) são biopolímeros biodegradáveis e biocompatíveis. Os PHAs são considerados uma solução possível como substitutos dos plásticos derivados do petróleo, podendo ser produzidos no âmbito do conceito de Biorefinaria utilizando resíduos como fonte de carbono. Este trabalho teve como objectivo o isolamento e a caracterização de bactérias produtoras de PHAs a partir de licor de cozimento ao sulfito ácido (HSSL), um sub-produto da indústria papeleira. Os isolamentos foram realizados partindo de uma cultura mista seleccionada para a acumulação de PHAs por imposição de ciclos de fome e fartura, utilizando alguns dos componentes do HSSL como substrato, nomeadamente a xilose e o ácido acético. Após repicagens sucessivas em meio sólido contendo HSSL, foi possível obter cinco isolados puros capazes de acumular PHAs. A pureza dos isolados foi avaliada através de coloração de Gram e análise FISH e a capacidade de acumulação de PHAs por coloração de Azul do Nilo. Duas estirpes foram identificadas como *Rhodococcus* spp. e três como *Pseudomonas* spp.. Um isolado de cada género foi seleccionado e estudado em termos de crescimento e capacidade de acumulação de PHAs, a partir de três fontes de carbono distintas (HSSL, ácido acético e xilose). Verificou-se que ambos os isolados, *Rhodococcus* spp. e *Pseudomonas* spp., foram capazes de crescer nos três meios e produziram PHAs. Contudo, ambas as estirpes apresentaram uma taxa específica de crescimento (μ_{max}) superior com HSSL como fonte de carbono, $0.212 \pm 0.0219h^{-1}$ e $0.251 \pm 0.0526h^{-1}$ respectivamente. Uma avaliação qualitativa da acumulação de PHAs utilizando coloração Azul do Nilo mostrou uma acumulação maior nos ensaios em que o ácido acético era a única fonte de carbono.

Numa tentativa de identificar algumas das espécies responsáveis pela acumulação de PHAs da cultura mista seleccionada pertencentes à classe dominante, *Alfaproteobactéria*, recorreu-se à construção de uma biblioteca de clones 16S rDNA. Foram identificadas as espécies *Novosphingobium* spp., *Sphingobium* spp e *Pleomorphomonas* spp.

keywords

Polyhydroxyalkanoates, Mixed Microbial Culture, HSSL, FISH, PCR, 16S rDNA

abstract

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible biopolymers. PHAs emerge as a possible solution as substitutes of petroleum based plastics, being produced under the Biorefinery concept, in which wastes and by-products of numerous industries may be used as carbon source.

This project aimed the isolation and characterization of organisms able to store PHAs from Hardwood Sulphite Spent Liquor (HSSL), a by-product of the pulp and paper industry. Isolation was performed from a Mixed Microbial Culture (MMC) selected under feast and famine conditions, using some components present in HSSL as substrates, such as acetic acid and xylose. Five pure isolates able to produce PHAs resulted from the successive streaking in solid medium containing HSSL. The purity of the isolates was evaluated through Gram staining and FISH analysis and the PHAs accumulation by Nile Blue staining. Two strains were identified as *Rhodococcus* spp. and three as *Pseudomonas* spp.. One isolate of each genus was selected and further studied in terms of growth and PHAs accumulation capability from three distinct carbon sources (HSSL, acetic acid and xylose). Both isolates, *Rhodococcus* spp. and *Pseudomonas* spp., were able to grow and use the three carbon sources as well as to produce PHAs. However, both strains showed a higher maximum specific growth rate (μ_{max}) when HSSL was used as carbon source, $0.212 \pm 0.0219 \text{ h}^{-1}$ and $0.251 \pm 0.0526 \text{ h}^{-1}$, respectively. A qualitative evaluation of the PHAs accumulation through Nile Blue staining exhibited a higher accumulation when acetic acid was used as sole carbon source.

In an attempt to identify some of the species responsible for PHAs accumulation of the selected MMC, belonging to the dominant class, *Alphaproteobacteria*, a 16S rDNA clone library was constructed. It was possible to identify *Novosphingobium* spp., *Sphingobium* spp. and *Pleomorphomonas* spp.

Contents

1. Introduction	1
2. State of the art	3
2.1 Plastics and Bioplastics.....	3
2.2 Polyhydroxyalkanoates (PHAs).....	3
2.2.1 Structure	4
2.2.2 Properties of PHAs	6
2.2.3 Applications of PHAs.....	7
2.2.3.1 Industrial applications.....	8
2.2.3.2 Agricultural applications.....	8
2.2.3.3 Medical applications	9
2.2.3.4 Other Applications	9
2.2.4 Commercially available PHAs and their applications.....	10
2.3 PHAs production process.....	11
2.3.1 Mixed Microbial Cultures	12
2.3.2 Low Value Substrates	14
2.3.2.1 Hardwood Sulphite Spent Liquor (HSSL).....	14
2.3.2.1.1 PHAs production by MMC from HSSL.....	16
2.4 Microbial Community Analysis of Selected MMC under ADF Conditions	17
2.5 Isolation of PHAs-Accumulating Bacteria from Activated Sludge.....	19
3. Methods and Materials	23
3.1 Culture	23
3.2 Culture Medium.....	23
3.2.1 HSSL pretreatment	23
3.2.2 Medium composition.....	23
3.3 Bacterial isolation	24
3.4 Analytical Methods.....	25
3.4.1 Carbon Sources Analysis.....	25
3.4.2 Biomass concentration.....	25
3.5 Sample fixation and storage.....	25
3.6 Gram Staining	26

3.7	Nile Blue Staining.....	27
3.8	FISH analysis.....	28
3.9	DNA extraction.....	29
3.10	PCR and Sequencing.....	30
3.11	Cloning of the 16S rDNA.....	31
4.	Results and Discussion.....	33
4.1	Isolation attempts using HSSL – based medium.....	33
4.1.1	Identification of isolates.....	38
4.2	Kinetic tests with isolates AF1 and AF3.....	41
4.2.1	Isolated strain AF1 – <i>Rhodococcus spp.</i>	41
4.2.2	Isolated strain AF3 – <i>Pseudomonas spp.</i>	44
4.2.2.1	PHAs accumulation.....	47
4.3	16S rDNA clone library.....	51
4.3.1.1	FISH analysis.....	57
5.	Concluding remarks.....	59
6.	Future Prospects.....	61
7.	Bibliography.....	63

Figures

Figure 1: Structure of PHAs granules from <i>Cupriavidus necator</i> (Rehm 2010).	4
Figure 2: General structure of polyhydroxyalkanoates (Hazer et al. 2012).	5
Figure 3: Schematic representation of PHAs biosynthesis from sugar catabolism, fatty acid β -oxidation and intermediary pathways (Gumel et al. 2012).	11
Figure 4: Representation of the pulp production process by acidic magnesium-based sulphite pulping of <i>Eucalyptus globulus</i> wood (Fernandes et al. 2012)	15
Figure 5: a) DGGE profile of the community structure of the activated sludge used as SBR inoculum (I) and their corresponding SBR selected sludge (II) (Dionisi et al. 2006); b) RT-PCR strategy applied for the identification of PHAs-accumulating organisms (Lemos et al. 2008).	18
Figure 6: Optic microscope Zeis Axioskop equipped with JVC TK-128OE Color Video Camera.	26
Figure 7: Epifluorescence microscope Olympus BX51, equipped with an Olympus XM10 camera.	27
Figure 8: MMC plated in solid medium containing HSSL (A); isolated strain AF2 in pure culture, in solid medium containing HSSL (B).	34
Figure 9: Microbial growth evolution, in $gVSS.L^{-1}$, and substrate consumption, in $gCOD.L^{-1}$, along the kinetic tests performed with the isolated strain AF1- <i>Rhodococcus</i> spp., in three different medium, each with a distinct carbon source: HSSL (C), acetic acid (B) and xylose (C).	43
Figure 10: Microbial growth evolution, in $gVSS.L^{-1}$, and substrate consumption, in $gCOD.L^{-1}$, along the kinetic tests performed with the isolated strain AF3- <i>Pseudomonas</i> spp., in three different medium, each with a distinct carbon source: HSSL (A), acetic acid (B) and xylose (C).	46
Figure 11: Overlap of FISH pictures. Green cells were only hybridized by EubMix probe and the red cells represent the cells hybridized by EubMix and Sph120 probes. Blue cells represent the remaining biomass that was not hybridized.	57

Tables

Table 1: Physical properties of specific PHAs and two synthetic polymers. Adapted from (Akaraonye et al. 2010).	6
Table 2: Three industrially available PHAs, the companies who produce them and their applications (Philip et al. 2007; Chanprateep 2010).....	10
Table 3: Eucalypt HSSL chemical composition (Xavier et al. 2010).	15
Table 4: PHAs production from bacterial isolates from activated sludge.....	20
Table 5: Concentration of nutrients in the culture medium.....	24
Table 6: Probes and their sequences used in FISH.....	28
Table 7: Probes and their sequences used in FISH.....	31
Table 8: Pictures of Gram and Nile Blue staining of the five isolates and the results of sequencing.	36
Table 9: Results obtained from the kinetic tests.....	41
Table 10: Images of Phase Contrast and Nile Blue staining of samples of AF1- <i>Rhodococcus spp.</i> , taken at different times of growth in liquid medium with three different carbon sources (HSSL, acetic acid and xylose).....	49
Table 11: Images of Phase Contrast and Nile Blue staining of samples of AF3- <i>Pseudomonas spp.</i> , taken at different times of growth in liquid medium with three different carbon sources (HSSL, acetic acid and xylose).....	50
Table 12: Representation of the taxonomic affiliations of the clones obtained.	51
Table 13: Studies reporting the identification or isolation of strains belonging to the genera obtained in the clone library, from activated sludge samples as well as their PHAs production ability (if reported).	55

Abbreviations

3HA	3-hydroxyalkanoate
3HB	3-hydroxybutyrate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
ADF	Aerobic Dynamic Feeding
C/N	Carbon to Nitrogen Ratio
CoA	Coenzyme A
COD	Chemical Oxygen Demand
DGGE	Denaturing Gradient Gel Electrophoresis
FISH	Fluorescence <i>In Situ</i> Hybridization
HSSL	Hardwood Sulphite Spent Liquor
lcl	Long Chain-Length
LS	Lignosulphonates
mcl	Medium Chain-Length
MMC	Mixed Microbial Culture
NREL	National Renewable Energy Laboratory
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HHD)	Poly(3-hydroxyhedecanoato)
P(3HOD)	Poly(3-hydroxyhedecanoato)
P(4HB)	Poly(4-hydroxybutyrate)
PBS	Phosphate Buffered Saline
PHAs	Polyhydroxyalkanoates
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SBR	Sequencing Batch Reactor
scl	Short Chain-Length
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solids
μ_{\max} (h ⁻¹)	Maximum Specific Growth Rate
t _d (h)	Duplication time
q _s	Substrate Specific Uptake Rate
Y _(X/S)	Yield of Biomass on Substrate

1. Introduction

Mankind has been relying on chemicals, polymers and fuels produced from fossil resources (petroleum, natural gas, and coal) ever since industrialization (Kamm et al. 2006; Gao et al. 2011; Pei et al. 2011). However, fossil fuels are not considered sustainable. A significant number of environmental and economic concerns arose from the massive scale processing of the current fossil resources by chemical industry. In addition to this, the depletion of fossil resources is expected in the near future (Kamm et al. 2006; Octave et al. 2009). Thus, it is essential to establish solutions to promote a sustainable development in the 21st century. One of the current approaches is the progressive transition to an economy based on renewable materials (e.g. biomass) as feedstock for the production of bio-products within the biorefinery concept (Kamm et al. 2006; Octave et al. 2009). The American National Renewable Energy Laboratory (NREL) defined biorefinery as “a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass” and that “the biorefinery concept is analogous to today’s petroleum refineries, which produce multiple fuels and products from petroleum” (Kamm et al. 2006; NREL 2012). Biorefineries would provide energy (biofuel, heat), molecules (fine chemistry, cosmetics, paramedical), materials (plastics, composites) as well as food ingredients (Octave et al. 2009).

Plastics have gained importance as a way to enhance life quality and comfort and are present in our everyday life in all sorts of forms, from disposable utensils to packaging (Khanna et al. 2005; Castilho et al. 2009). Their use is related to their properties such as strength, lightness, durability and resistance to degradation. However, some of these qualities are now their major problem (Khanna et al. 2005) since make their disposal difficult (Castilho et al. 2009). Accumulation of microscopic plastic debris at sea is particularly alarming as well as the exponentially increasing need of landfill for municipal solid waste disposal (Gomez et al. 2012).

Polyhydroxyalkanoates (PHAs) are polymers produced biologically, with similar properties to synthetic plastics. PHAs are biodegradable, biocompatible and may be produced from renewable sources, thus offering a solution to the environmental hazards displayed by conventional plastics (Khanna et al. 2005; Gumel et al. 2012).

Isolation and characterization of PHAs-accumulating bacteria from HSSL

This project aim to study the isolation and characterization of organisms from a mixed microbial culture selected under feast and famine conditions able to store PHAs from the different carbon sources present in Hardwood Sulphite Spent Liquor (HSSL), a by-product of the pulp and paper industry (Caima S.A.).

The microorganisms were isolated from a PHAs-accumulating mixed microbial culture (MMC) in solid medium containing HSSL. The isolates were characterized using microscopy and molecular biology techniques and their identification was attempted. Additionally, a 16S rDNA clone library was constructed in order to identify the other PHAs-accumulating bacteria present in the MMC. Finally the consumption of different carbon sources, present in HSSL, such as acetic acid and xylose, by the isolates was studied, and the growth kinetic parameters determined.

2. State of the art

2.1 Plastics and Bioplastics

Petrochemical-based plastics are nowadays one of the most applied materials, being used in domestic, medical and industrial applications. The rather low price and versatile qualities of strength, lightness, durability and resistance to degradation are the main reasons for their huge success (Zinn et al. 2001; Khanna et al. 2005; Keshavarz et al. 2010). However, these qualities became a major disadvantage of their use. Adequate methods for plastics disposal are problematic (Castilho et al. 2009) and their accumulation in the environment is considered a world-wide problem (Khanna et al. 2005). Degradation rates in landfills are extremely low (Castilho et al. 2009) and incineration is expensive and may generate toxic by-products, such as hydrogen cyanide produced from the combustion of acrylonitrile-based plastics (Khanna et al. 2005; Castilho et al. 2009). Recycling is a time-consuming disposal method and the presence of a wide variety of additives limits the use of the recycled materials (Khanna et al. 2005; Castilho et al. 2009). In addition to this, petroleum reserves are finite, therefore a new source of durable materials is needed (Akaraonye et al. 2010).

Bioplastics may provide a solution to the environmental hazards brought by traditional plastics. Efforts are being made in order to find polymers of biological origin, biodegradable and with industrial application (Zinn et al. 2001; Castilho et al. 2009). Bioplastics like, polyhydroxyalkanoates (PHAs), polylactic acid (PLA) and other polymers derived from renewable resources, are already available in the market (Pei et al. 2011).

2.2 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates are polymers of hydroxyalkanoic acids produced by prokaryotic microorganisms (*Bacteria* and *Archaea*) accumulated in the cell cytoplasm as insoluble spherical inclusions, to serve as carbon and energy storage. Lemoigne was the

first to clarify the chemical composition of these intracellular granules in 1926, although numerous microbiologists had reported their presence in bacterial cells (Lemoigne 1926; Khanna et al. 2005). Lemoigne identified poly-3-hydroxybutyrate (P(3HB)) from *Bacillus megaterium* (Lemoigne 1926). Since then, over 90 genera of microorganisms PHAs-producers have been identified, in both aerobic and anaerobic environments (Zinn et al. 2001).

The PHAs granules are usually between 0.2 to 0.5 μm in diameter (Sudesh et al. 2000). Their structure, as represented in Figure 1, consists of a PHA core, amorphous and hydrophobic, delimited by a boundary phospholipid monolayer with attached proteins involved in PHAs metabolism, that include PHA synthase, phasins, depolymerizing enzymes and regulatory enzymes (Grage et al. 2009; Jendrossek 2009).

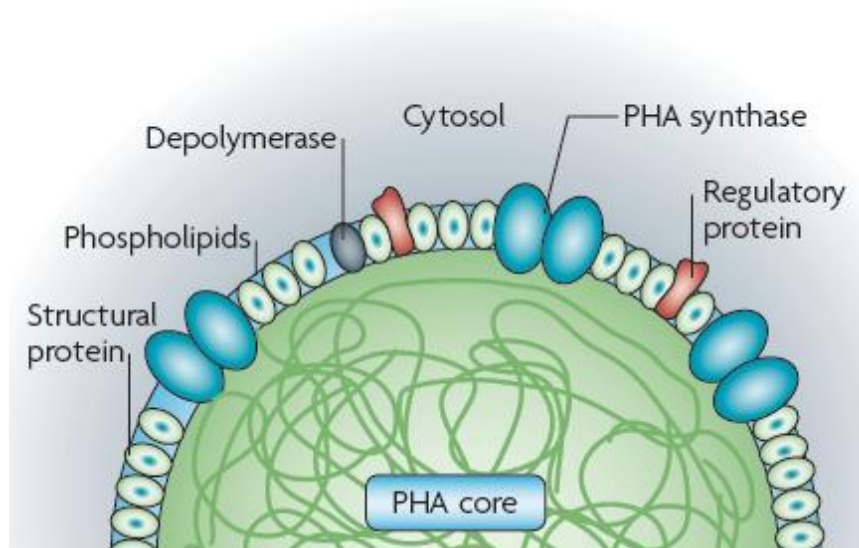


Figure 1: Structure of PHAs granules from *Cupriavidus necator* (Rehm 2010).

2.2.1 Structure

Multiple PHAs have been identified, with a great variety in terms of type and ratio of monomers (Castilho et al. 2009). Over 150 known hydroxyalkanoic acids were recognized as monomers of these polymers (Rehm 2010). P(3HB) is the most abundant PHAs family member.

PHAs structure is shown in Figure 2. Monomers are linked through an ester bond between the carboxyl group of one hydroxyalkanoic acid and the hydroxyl group of the next one. This reaction is catalyzed by the PHA synthase of the microorganism. The number of monomers (X) can vary from 100 to 30000. The group R represents alkyl groups and varies from methyl (C1) to tridecyl (C13). The hydroxyalkanoic acid units are all in the R configuration because of the stereo-specificity of PHA synthase, therefore PHAs are optically active (Sudesh et al. 2000).

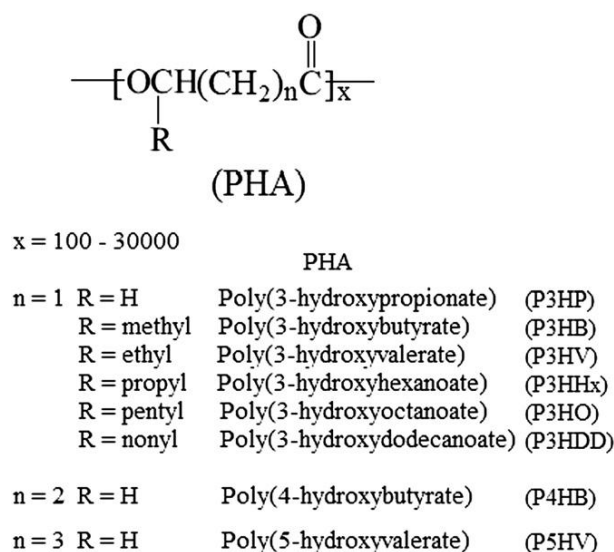


Figure 2: General structure of polyhydroxyalkanoates (Hazer et al. 2012).

Structurally, PHAs are classified on the basis of number of carbon atoms of monomers. There are three classes: short chain length PHAs (scl-PHAs), in which the monomers have between 3 and 5 carbon atoms, medium chain length PHAs (mcl-PHAs), consisting of monomers with 6 to 14 carbon atoms, and long chain length PHAs (lcl-PHAs), monomers with over 14 carbon atoms (Singh et al. 2008)

These polymers are also classified according to the type of monomeric units: homopolymers, copolymers and terpolymers (Keshavarz et al. 2010). Poly(3-hydroxybutyrate-co-hydroxyvalerate) (P3HB-co-3HV) is an example of a copolymer that belongs to scl-PHAs and poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate) (P3HHx-co-3HO) to mcl-PHAs (Akaraonye et al. 2010).

2.2.2 Properties of PHAs

The physical properties of PHAs, such as melting temperature, glass transition temperature and crystallinity (stiffness and flexibility), depend on the number of monomers, their compositions, side chain length and functional group (Akaraonye et al. 2010; Nitschke et al. 2011).

The physical properties of six different PHAs and two synthetic polymers, polypropylene and polystyrene, are displayed in Table 1. As the length of the side chain of PHAs increases, the physical properties change from glassy state to more soft and sticky material (Hazer et al. 2012). Scl-PHAs are crystalline polymers, rather stiff, with high melting points, low glass transition temperatures, and some of them have higher tensile strength than synthetic polymers. On the other hand, mcl-PHAs are thermoplastic elastomers with low crystallinity and tensile strength but high elongation to break and have lower melting points and glass transition temperatures, when compared to scl-PHAs (Akaraonye et al. 2010).

Table 1: Physical properties of specific PHAs and two synthetic polymers. Adapted from (Akaraonye et al. 2010).

Polymer	Melting temperature (°C)	Glass transition temperature (°C)	Young's modulus (GPa)	Elongation to break (%)	Tensile strength (MPa)
P(3HB)	180	4	3.5	5	40
P(4HB)	53	-48	149	1000	104
P(3HB-co-20% 3HV)	145	-1	1.2	50	20
P(3HB-co-16% 4HB)	150	-7	-	444	26
P(3HB-co-10% 3HHx)	127	-1	-	400	21
P(3HB-co-6% 3HD)	130	-8	-	680	17
Polypropylene	176	-10	1.7	400	34.5
Polystyrene	240	100	3.1	-	50

The incorporation of monomer units to form copolymers can also improve physical properties (Khanna et al. 2005; Castilho et al. 2009). Khanna et al. (2005) reviewed the structure and properties of copolymers of different monomers. These authors concluded that as the fraction of 3HV increased, the copolymer became tougher (impact strength

increased), more flexible (Young's modulus decreased), the elongation to break increased and the melting temperature decreased without affecting degradation temperature thus allowing thermal processing of copolymer melts without thermal degradation. Therefore, material properties can be controlled by adjusting the fraction of monomers during the fermentation (Khanna et al. 2005).

Biocompatibility and biodegradability are the two other main properties of PHAs, responsible for the extensive attention these biopolymers have received (Akaraonye et al. 2010). Biocompatibility is characterized by the absence of any toxic compound generated during polymer degradation, as well as by the shape and surface porosity of the material (Castilho et al. 2009). The biocompatibility of several specific PHAs has been studied and verified, including P(3HB), P(3HB-co-3HV), P(4HB), P(3HO) and P(3HB – co-3HHx) (Chen et al. 2005; Akaraonye et al. 2010).

PHAs are biodegradable once they can be degraded producing carbon dioxide and water, in aerobic environments, or methane, in anaerobic conditions, by a large variety of microorganisms when disposed in numerous ecosystems, such as soil, sewage, sea and lake water (Lee 1996). The enzyme responsible for this degradation is the extracellular PHA depolymerase, produced by the degrading microorganisms. This enzyme converts the polyester into water-soluble oligomers and monomers that are used as a carbon source within the cells (Reddy et al. 2003). The biodegradability of PHAs is influenced by the properties of the polymer such as stereo-regularity, crystallinity, composition and accessibility of their surface to PHA depolymerizing enzymes, and by environmental conditions, temperature, moisture level, pH and nutrient supply (Sudesh et al. 2000). For example, PHA copolymers containing 4HB monomer unit degrade more rapidly than P(3HB) or P(3HB-co-3HV) copolymers (Reddy et al. 2003).

2.2.3 Applications of PHAs

The very particular properties of PHAs, such as physical properties similar to those of synthetic polymers, biodegradability and biocompatibility, resulted in a huge commercial interest towards these biopolymers (Grage et al. 2009; Akaraonye et al. 2010). The major advantage of PHAs is that both the physical properties and the rate of

degradation of PHAs can be altered by changing the bacterial source of the polymer and the corresponding fermentation conditions used (Akaraonye et al. 2010). This fact allows for tailoring PHAs composition according to the properties needed for a specific application.

If initially PHAs were used only to make everyday articles, such as shampoo bottles and packaging materials, nowadays their range of applications is much wider, both in variety and specialization (Philip et al. 2007). The applications of PHAs may be divided in three areas: industrial, agricultural and medical, among others.

2.2.3.1 Industrial applications

PHAs have been extensively tested to be used in industry (Philip et al. 2007). PHAs can be used as substitutes for many conventional petrochemical products in applications including molded goods, foils, films and diaphragms and to produce food additives, flushables, non-woven fabrics, combs and pens (Philip et al. 2007; Akaraonye et al. 2010). Rubber made of PHAs might be applied to paper or cardboard to form a water-resistant layer (Nitschke et al. 2011). Furthermore, PHAs can be employed as sources for the synthesis of enantiomerically pure chemicals such as hydroxyalkanoic acids and as raw materials for the production of latex paints (Akaraonye et al. 2010). Due to their piezoelectric nature, it is also possible to use PHAs to make pressure sensors for keyboards, stretch and acceleration measuring instruments, shock wave sensors and lighters (Philip et al. 2007).

2.2.3.2 Agricultural applications

PHAs can be used as protection films for agricultural purposes, for long term release of fertilizers, insecticides and herbicides, as well as for the stabilization of commercial bacterial inoculants (Philip et al. 2007). Bacterial cultures used in inoculant preparations for agriculture purposes must be able to withstand stressful environments. The use of PHAs significantly improved the shelf life, efficiency and reliability of commercial inoculants (Nitschke et al. 2011).

2.2.3.3 Medical applications

Many studies using various animal models have demonstrated that different PHAs, namely P(3HB), P(3HB-co-3HV), P(4HB), P(HO) and P(3HB-co-HHx), possess the characteristics needed for implant applications and controlled drug release, namely, biodegradability, biocompatibility and thermoprocessibility (Chen et al. 2005). Therefore, PHAs have been used for the production of temporary stents, bone plates, nails and screws, surgical sutures and other medically relevant materials (Nitschke et al. 2011).

According to the requirements for the different applications, PHAs can be blended, surface modified or composited with other PHAs and other polymers, enzymes or even inorganic materials to further adjust their mechanical properties or biocompatibility. The many possibilities to tailor-made PHAs for medical implant applications have shown this class of materials to have a bright future as tissue engineering materials (Chen et al. 2005).

2.2.3.4 Other Applications

A recent development is the potential use of PHAs granules, formed inside recombinant bacterial cells, as tailor-made functionalized micro- and nano-beads in which specific proteins attached to the PHA core have been engineered to display various protein functions. The application performance of engineered PHA beads in high-affinity bioseparation, enzyme immobilization, protein production, diagnosis and as an antigen delivery system has been demonstrated, and the technology is now being commercialized (Rehm 2010).

Furthermore, after used as bioplastic, PHAs monomers may be further methyl esterified to become hydroxyalkanoate methyl ester, which produces combustion heats comparable to ethanol, and so might be used as biofuels (Gao et al. 2011).

2.2.4 Commercially available PHAs and their applications

Several commercial brands of PHAs are currently available and some of them are summarized in Table 2. PHAs are becoming price-competitive with petroleum-based polymers. The price of synthetic plastics is around 1€/kg, for polypropylene. In 2006, PHA cost was about 3.5 to 5.0 €/kg. Nevertheless this price has significantly decreased (Table 2) (Chanprateep 2010). Among PHAs-manufacturing companies, the main company with a large production is the USA biotech company Metabolix, Inc. in Cambridge, Massachusetts (Chanprateep 2010).

Table 2: Three industrially available PHAs, the companies who produce them and their applications (Philip et al. 2007; Chanprateep 2010).

Polymer	Trade name	Manufactures	Price (kg⁻¹) (in 2010)	Applications
P(3HB) and P(HB-co-HV)	Biomer®	Biomer Inc. (Germany)	3.0 – 5.0 €	-polymer pellets sold commercially for use in classical transformation processes -production of articles (combs, pens and bullets)
P(3HB)	Mirel™	Metabolix (USA)	1.50 €	-coat paper and paperboards -injection, blow moulding, film production and for electric and electronic packaging -produce shampoo bottles, motor oil bottles and disposable razors
P(3HB-co-3HHx)	Nodax™	Procter and Gamble (USA)	2.50 €	-available as foam, fibres or non-wovens, films and latex -produce flushables, medical surgical garments, upholstery, carpet, packaging, compostable bags and lids or tubs for thermoformed articles -coating for fertilizers, herbicides or insecticides.

The biosynthesis of PHAs from sugars is the best known pathway among the PHAs biosynthetic pathways. It normally begins with glycolysis of the sugar to pyruvate, which is converted to acetyl-CoA via the pyruvate dehydrogenase (PDH) oxidation pathway. Then, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA, by the action of β -ketothiolase, an enzyme encoded by the *phaA* gene. Acetoacetyl-CoA is reduced by acetoacetyl-CoA reductase (*phaB*) to form the monomer of (R)-3-hydroxyacyl-CoA, the building block of PHAs. Finally, PHA synthase (*phaC*) polymerizes the monomers to PHAs (Philip et al. 2007; Gumel et al. 2012). The three different pathways for PHAs biosynthesis are represented in Figure 3.

The current industrial production processes are mainly based on the use of pure cultures of microorganisms in their wild form, such as *Cupriavidus necator*, *Alcaligenes latus*, or using genetically modified strains, such as recombinant *Escherichia coli*, by cloning the PHA synthase genes from many microorganisms, including *C. necator* (Lemos et al. 2006; Chen 2009; Akaraonye et al. 2010; Chanprateep 2010). *C. necator* has been the most commonly used wild type strain for the industrial production of P(3HB), P(3HB-co-4HB) and P(3HB-co-3HV). *E. coli* has been favored in the choice as host due to its ability to grow fast, to achieve high cell density from several inexpensive substrates and simple polymer purification (Lemos et al. 2006; Akaraonye et al. 2010).

Over the past few years, the search for alternative processes in order to reduce the PHAs production costs has significantly increased. The use of complex low cost carbon substrates and mixed microbial cultures has been proposed to reduce these costs (Serafim et al. 2008; Gumel et al. 2012).

2.3.1 Mixed Microbial Cultures

In order to reduce the costs of industrial PHAs production, the choice of the microorganism used depends on several factors that include the ability of cells to use inexpensive carbon sources, the growth rate, the polymer productivity, the quality and amount of PHAs obtained and the cost of the downstream processes (Chen 2009; Chanprateep 2010).

An interesting alternative to pure cultures is the use of mixed microbial cultures (MMC), such as activated sludge from wastewater treatment plants. The use of MMC affords the advantage that sterile conditions are not needed, thus saving the energy and equipment costs (Serafim et al. 2008; Villano et al. 2010). PHAs accumulation by mixed cultures was first detected in wastewater treatment plants designed for biological phosphorus removal (EBPR), which operate with alternated anaerobic and aerobic cycles (Serafim et al. 2008).

Activated sludge with PHAs storage capacity may also be observed in aerobic wastewater treatment plants, in a process known as “aerobic dynamic feeding” (ADF) or “feast and famine”. In this process the activated sludge undergoes consecutive periods of external accessibility (feast) and unavailability (famine). PHAs storage occurs due to an internal growth limitation that results from insufficient intracellular components such as enzymes or RNA (Dias et al. 2006; Serafim et al. 2008). Under such conditions, microorganisms able to store the substrate during the feast phase possess a competitive advantage over the others, as the stored polymer can act as an internal carbon source during the famine phase. Therefore, under these conditions, activated sludge can be enriched with PHAs-accumulating microorganisms and its overall PHAs-storage potential is greatly increased (Serafim et al. 2008; Villano et al. 2010).

PHAs production processes by mixed cultures are usually operated in two or three steps, depending on the type of substrate used as feedstock (Dias et al. 2006). The three-step process consists firstly of a fermentative stage. In this stage occurs acidogenic fermentation, thus converting the biodegradable waste into a high-concentration mixture of acetate and other volatile fatty acids (VFAs), the substrates used by MMC for PHAs storage. The second step consists of an enrichment stage. Activated sludge process is performed at high organic load imposing periodic feeding in a fully aerobic SBR, in order to enrich and produce sludge with a high storage response. Finally, an accumulation step is performed. The storage response of the produced sludge is aerobically exploited in order to increase the amount of polymer produced (Dias et al. 2006; Villano et al. 2010). The two step process lacks the first stage described (Dias et al. 2006).

2.3.2 Low Value Substrates

The selection of a suitable substrate is an important factor to optimize PHAs production since it affects the final cell content, composition and properties of the polymer. 40% of total operating expenses of PHAs production are related to the raw materials, and more than 70% of this cost is attributed to the carbon source. Because of these costs there has been considerable interest in the use of cheap carbon substrates (Salehizadeh et al. 2004; Akaraonye et al. 2010).

Several agricultural or industrial waste materials have been explored as cheap carbon and nitrogen sources for PHAs production. Recycling of wastes generated from agriculture and industries for PHAs production is not only crucial for reduction of production costs but also for waste management. Process economics have revealed that the use of inexpensive and renewable carbon substrates as PHAs carbon feedstock can contribute to as much as 40-50% reduction in the overall production cost (Nitschke et al. 2011). The various cheap carbon sources already tested include whey, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater, palm oil mill effluent and subproducts of lignocellulosic biomass processing, such as hardwood sulphite spent liquor, HSSL (Keenan et al. 2006; Akaraonye et al. 2010).

2.3.2.1 Hardwood Sulphite Spent Liquor (HSSL)

HSSL is a by-product resulting from the sulphite pulping of hardwood, such as *Eucalyptus globulus*, and is usually concentrated and burned for chemicals and energy recovery (Figure 4) (Lawford et al. 1993; Marques et al. 2009). Acidic sulphite magnesium-based pulping of *Eucalyptus globulus* wood occurs usually around 130°C and a pH of 1.5 (Marques et al. 2009).

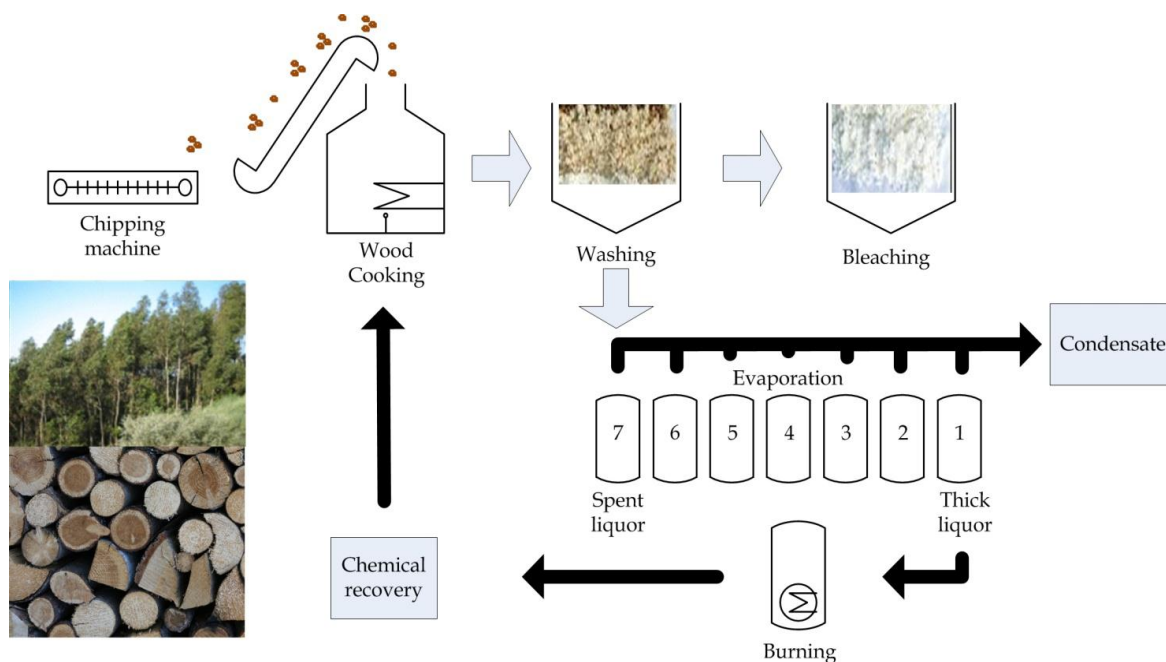


Figure 4:Representation of the pulp production process by acidic magnesium-based sulphite pulping of *Eucalyptus globulus* wood (Fernandes et al. 2012) .

The chemical composition of HSSL is listed in Table 3. The main components are lignosulphonates and sugars, with concentrations around 78 g L^{-1} and 50 g L^{-1} , respectively. The sugars were obtained from the partial hydrolysis of hemicelluloses (glucuronoxylan, glucomannan and glucans), and the D-Xylose is present at highest concentration, in both monomeric (about 70 to 75%) and oligomeric (about 25 to 30%) forms (Marques et al. 2009; Xavier et al. 2010). Due to this fact, HSSL is a potential substrate for bioprocessing (Marques et al. 2009).

Table 3: Eucalypt HSSL chemical composition (Xavier et al. 2010).

Components	Concentration (g L^{-1})
Lignosulphonates	78.2 ± 0.6
Acetic Acid	8.2 ± 0.3
Furfural	<0.1
Ash	19.8 ± 0.2
D-Xylose	24.6 ± 0.5
D-Mannose	8.5 ± 0.9
L-Arabinose	7.8 ± 0.3
D-Galactose	4.5 ± 0.1
D-Glucose	2.3 ± 0.1
L-Rhamnose	1.6 ± 0.3
L-Fucose	0.4 ± 0.3

However, the acidic hydrolysis of lignocellulosic materials also releases several compounds derived from sugar and degradation of lignin, namely furfural, hydroxymethylfurfural, acetic acid, syringic acid, and others (Mussatto et al. 2004; Marques et al. 2009; Xavier et al. 2010). Some of these compounds, such as furfural and acetic acid, are toxic to fermentative microorganisms and inhibit their metabolism, which is a major factor limiting HSSL bioprocessing (Mussatto et al. 2004). Furfural is a degradation product from the hydrolysis of pentose sugars, namely xylose. It can inhibit cells and affect the specific growth rate and cell-mass yield depending on its concentration in the fermentation medium (Palmqvist et al. 2000). Acetic acid derives from acetyl groups present in the hemicellulose. According to the fermentative process conditions the toxicity of this acid differs (Mussatto et al. 2004). When the medium pH is low, acetic acid is in the undissociated form and is capable to diffuse across the cell membrane (liposoluble). Inside the cell it dissociates and accumulates in the cytoplasm, causing the decrease of internal pH, inhibiting cell activity and even causing death (Lawford et al. 1993; Mussatto et al. 2004).

The use of HSSL as raw material for the production of value added products fits well to the biorefinery concept, developed to decrease the dependence from fossil resources and to improve the economic sustainability of pulp mills (Marques et al. 2009; Xavier et al. 2010). The production of several products has been tested using this by-product as substrate, which includes bioethanol (Lawford et al. 1993; Xavier et al. 2010; Pereira et al. 2012), single cell protein (Pereira et al. 2012), bacterial cellulose (Carreira et al. 2011) and PHAs (Queirós 2012).

2.3.2.1.1 PHAs production by MMC from HSSL

The possibility of using HSSL as a substrate for PHAs production by a MMC was studied by Queirós (2012). The author performed the selection of PHAs-accumulating organisms from activated sludge collected in a wastewater treatment plant, which was submitted to ADF in Sequencing Batch Reactor (SBR) using HSSL as substrate. The selected MMC reached a maximum PHAs content of 67.6% and consumed the total content of acetic acid and a small part of xylose (Queirós 2012).

2.4 Microbial Community Analysis of Selected MMC under ADF Conditions

PHAs productivity and content obtained by activated sludge has been generally lower than the obtained by pure cultures. One of the main factors on the development of a competitive process for PHAs production with MMC is the selection of organisms with high storage capacity (Lemos et al. 2008). Therefore, efforts have been made towards the identification of microorganisms responsible for PHAs storage, including production by selected activated sludge under highly dynamic conditions to promote as much storage as possible (Dionisi et al. 2006; Serafim et al. 2006; Lemos et al. 2008; Jiang et al. 2011; Jiang et al. 2012; Moita et al. 2012; Queirós 2012).

Dionisi et al. (2006), applied molecular methods to identify PHAs-accumulating organisms in an ADF reactor. The authors used denaturing gradient gel electrophoresis (DGGE) and performed 16S rRNA (ribosomal ribonucleic acid) clone library (Figure 5). The most represented group was *Betaproteobacteria* (*Thauera*, *Alcaligenes*, *Comamonas*, *Achromobacter*). The second phylogenetic group characterizing the SBR sludge was *Gammaproteobacteria* (*Kluyvera*, *Pseudomonas*, *Acinetobacter*) and *Alphaproteobacteria* (*Xantobacter*, *Curtobacterium*) were also present. It was found that *Thauera* genus was the dominant one and was the first time it was described as PHAs-producer. Fluorescence *In Situ* Hybridization (FISH) method in combination with Nile Blue staining was employed by Serafim et al. (2006), for the characterization of the microbial population in two reactors under ADF conditions, one adapted to acetate and the other to propionate. The results showed that the main PHAs-producers belonged to *Betaproteobacteria* and *Alphaproteobacteria*, and the main responsible for the PHAs storage was affiliated to the genus *Azoarcus* (*Betaproteobacteria*). Lemos et al. (2008) determined the PHAs storing bacteria selected under ADF conditions, using propionate or acetate as carbon source. The authors applied reverse transcriptase-polymerase chain reaction (RT-PCR) on micromanipulated cells and confirmed by FISH. Four genera were detected, *Amaricoccus*, *Azoarcus*, *Thauera* and *Paracoccus*, being the latter rarely present.

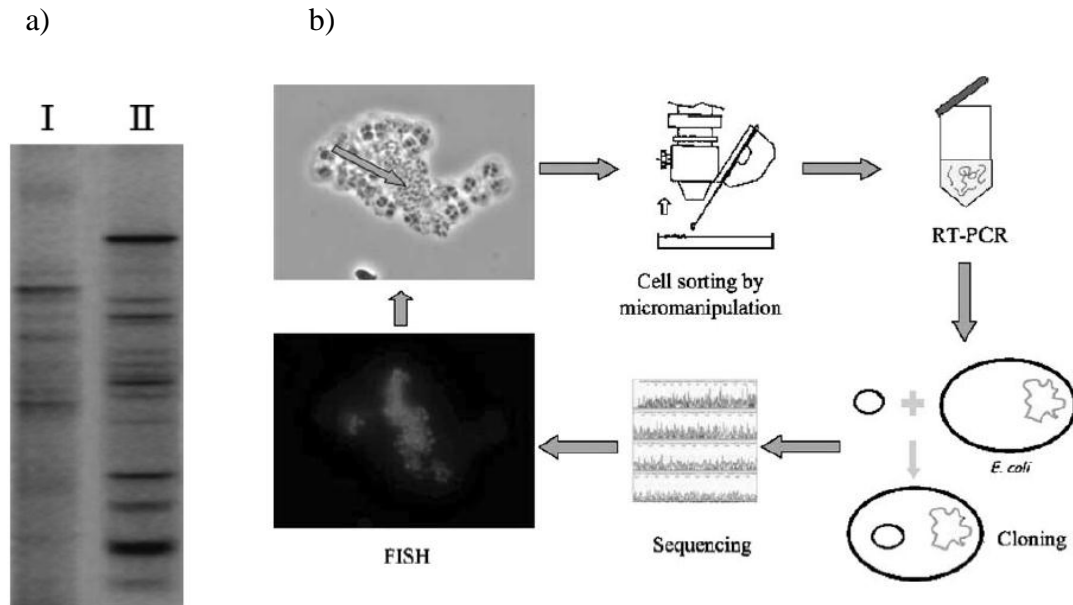


Figure 5: a) DGGE profile of the community structure of the activated sludge used as SBR inoculum (I) and their corresponding SBR selected sludge (II) (Dionisi et al. 2006); b) RT-PCR strategy applied for the identification of PHAs-accumulating organisms (Lemos et al. 2008).

The microbial community analysis of studies of PHAs production by selected activated sludge using complex substrates has also been performed. Jiang et al. (2011) used lactate as carbon source and *Plasticicumulans acidivorans* and *Thauera selenatis* were identified as the predominant bacterial species. In 2012, Jiang and co-workers, using paper mill wastewater, found a large amount of *P. acidivorans*, which was the main PHAs-producer, which accounted for 56% of the biomass, and a flanking population, which exhibited limited PHAs producing capacity (Jiang et al., 2012). Moita et al. (2012) used bio-oil from the fast-pyrolysis of chicken beds as substrate in a SBR under ADF conditions and observed the dominance of *Betaproteobacteria* over the *Alpha* and *Gammaproteobacteria* using FISH. *Thauera* genus was found to be the dominant genus and *Amaricoccus* and *Zooglea* were also detected (Moita et al., 2012). Finally, Queirós (2012) after the selection of PHAs-accumulating organisms using HSSL under ADF conditions analyzed the selected culture by FISH. The dominant bacteria belonged to *Alphaproteobacteria*, but *Beta* and *Gammaproteobacteria* were also present. At genus level were detected *Paracoccus* and *Deffluvicoccus*, both belonging to *Alphaproteobacteria*.

2.5 Isolation of PHAs-Accumulating Bacteria from Activated Sludge

Numerous researchers have attempted to isolate PHAs producing microorganisms from various sources aiming to discover and identify novel species with high PHAs production capacity (Sangkharak et al. 2012). The use of naturally producing microorganisms is economical as there is great potential for producing PHAs in low-cost substrates, which can reduce PHA production costs (Thirumala et al. 2010).

In addition to the microbial community analysis, in order to have a better understanding on the microbial strains of activated sludge responsible for PHAs accumulation, several studies have performed the isolation of PHAs-accumulating bacteria from activated sludge. The isolations were generally performed using plating techniques and the screening of the PHAs-accumulating microorganisms by Nile Blue staining. The isolates were identified, by biochemical and 16S rRNA sequence analysis and phylogenetic studies, and their capacity of PHAs production evaluated (Table 4).

The first successful isolation of PHAs-accumulating bacteria from activated sludge was reported by Liu et al. in 2000. The activated sludge was collected from an EBPR system and was enriched in an anaerobic-oxic system fed with acetate. The isolates were able to accumulate the copolymer P(3HB-co-3HV) (Liu et al. 2000).

The possibility of PHAs production by activated sludge isolates using food wastes as substrates was performed in 2001 and 2002. Law and co-workers (2001) isolated a bacteria related to *Bacillus megaterium* able to use both food wastes and accumulate P(3HB), whereas the *Klebsiella* isolated by Wong et al. in 2002 produced P(3HB-co-HV) copolymers with different monomer compositions (Table 4).

Table 4: PHAs production from bacterial isolates from activated sludge.

Reference	Isolated Bacteria from Activated Sludge	Carbon source	Polymer produced	PHA yield ¹ (%)	Fermentation Conditions
(Law et al. 2001)	Related to <i>Bacillus megaterium</i>	Malt waste Soy waste	P(3HB) P(3HB)	- -	Batch: 3.7 L bioreactor 37°C and pH 7.0
(Wong et al. 2002)	<i>Klebsiella</i> spp.	Malt waste Soy waste	P(3HB-co-3HV) ratio: 93:7 P(3HB-co-3HV) ratio: 79:21	- -	- -
(Liu et al. 2002)	<i>Sphaerotilus natans</i>	Glucose	P(3HB-co-3HV) ratio: 30:70	15.0	Fed-batch: 10 L bioreactor; 30°C; 300 rpm; 0.5 (v/v) aeration
(Akar et al. 2006)	<i>Micrococcus phosphovorans</i> (DMS 10555)	Glucose Glucose Acetate	P(3HB) P(3HB) P(3HB)	23.97 25.91 23.23	Batch: 2 L bioreactor; 30°C, 250 rpm Batch, Anaerobic- aerobic cycles; 30°C
(Kourmentza et al. 2009)	<i>Pseudomonas</i> sp. ²	VFAs mixture (Acetate, propionate and butyrate), with N limitation VFAs mixture, with N and O ₂ limitation	P(3HB) P(3HB)	23.77 50.61	Batch: 1 L Erlenmeyer Flasks; room temperature; 250 rpm
	<i>Pseudomonas</i> sp. ³	VFAs mixture, with N limitation VFAs mixture, with N and O ₂ limitation	P(3HB) P(3HB)	13.58 11.22	
(Thirumala et al. 2010)	<i>Bacillus</i> sp. 112A	Glucose Sucrose Starch	P(3HB) P(3HB) P(3HB)	67.73 58.50 50.50	Batch: 500 mL Flask; 30°C; 150 rpm
(Chen et al. 2011)	<i>Gamma proteobacterium</i> WD-3	Sodium butyrate	P(3HB-co-3HV) ratio: 35:65	45.0	Fed-batch: 250 mL Erlenmeyer flask; 30°C; 180 rpm
(Venkateswar Reddy et al. 2012b)	<i>Pseudomonas otitidis</i>	Synthetic acids Acidogenic effluent	P(3HB-co-3HV) P(3HB-co-3HV)	58.0 54.0	Batch: flasks; 120 rpm; 28°C

¹PHA yield is given in terms of PHA content (% of cell dry weight);

² *Pseudomonas* sp. with 99% similarity with *Pseudomonas putida* ATCC 17390, *Pseudomonas* sp. strain ONBA and *Pseudomonas* sp. strain HR13;

³ *Pseudomonas* sp. with 99% similarity with *Pseudomonas putida* ATCC 17453 and *Pseudomonas putida* ATCC 17514.

The production of the copolymer P(3HB-co-3HV) from inherent bacteria in activated sludge was further evaluated due to the advantage of production of the copolymer once PHAs copolymers composed of primarily 3HB with a fraction of longer chain monomers, such as 3HV, are more flexible and tougher plastics, and such plastics typically have a wider range of applications. Liu et al. (2002) isolated *Sphaerotilus natans* and investigated its ability to produce P(3HB-co-HV) using glucose and peptone, or by adding

valeric acid or sodium propionate as precursor and concluded that the highest 3HV content of the copolymer was 70% using glucose as carbon source and sodium propionate as precursor. Chen and co-workers, in 2011, isolated the *Gamma proteobacterium* WD-3 strain. The effects of altering of the C/N ratio (mol mol^{-1}) and changing in the carbon source (propionate, acetate and butyrate) on PHAs production were investigated in batch experiments. The highest PHAs content (22%) was obtained for a C/N ratio of 35. The use of different carbon sources produced a different combination of monomers in the PHAs, and the authors concluded that this strain could accumulate PHAs containing both the 3HB and 3HV monomers in the presence of a single carbon source. Finally, the authors performed a fed-batch experiment, with a C/N ratio of 35, in which the maximum PHAs accumulation was 45% of cell dry weight and the proportion of 3HV occupied one-third of the PHA end product. Venkateswar Reddy et al. (2012a) performed the isolation of several microorganisms involved in PHAs accumulation and waste treatment, after the enrichment of activated sludge under ADF conditions. The authors selected over 30 phenotypically different colonies, and 7 of these isolates were further characterized. The dominant organisms were related to phylum *Firmicutes*, and were identified as *B. subtilis*, *B.adius*, *B. tequilensis*, *Staphylococcus arlettae* and *Enterococcus italicus*. The other two isolates belonged to *Gamaproteobacteria*, *Serratiaureilytica* and *Pseudomonas otitidis* (Venkateswar Reddy et al. 2012a). PHAs production from the isolated *Pseudomonas otitidis* was further investigated, using two substrates, synthetic acids and acidogenic effluent from biohydrogen reactor. The results obtained revealed that this isolate showed ability to grow and accumulate PHAs (Table 4) with simultaneous waste remediation (Venkateswar Reddy et al. 2012b).

The production of homopolymer P(3HB) has also been studied. PHAs production by the isolated *Micrococcus phosphovorans* was studied by Akar et al. (2006) from an EBPR system. The authors investigated the PHA storage behavior of the isolate under different growth conditions and using different carbon sources. Two different strategies were employed, first batch-growth systems using chemically defined growth media with glucose as only carbon source followed a batch-growth system with anaerobic-aerobic cycles and varying concentrations of glucose or acetate as the sole carbon sources, similar to EBPR processes. After evaluation of all the experiments, the main polymer produced was P(3HB) with yields between 20-30% of cell dry weight and the highest P(3HB)

production was observed in batch-growth similar to EBPR with glucose as carbon source. Kourmentza et al (2009) isolated two *Pseudomonas* sp. from an enriched MMC by alternating cycles between limitation of carbon and nitrogen substrates. The isolates were studied in terms of PHAs accumulation under both nitrogen limitation and simultaneous nitrogen and oxygen limitation conditions, with a synthetic medium with volatile fatty acids as carbon source, and the results compared to the use of the mixed culture. The authors concluded that the enriched culture was more promising for PHAs production when compared to the two isolates, from that same mixed culture. However, the high percentage of PHAs accumulation in the first isolate (50.61%), under nitrogen and oxygen limitation indicated that this specific strain could lead to quite high yields under properly selected limiting conditions. Thirumala et al. (2010) studied the P(3HB) production by an isolated bacteria belonging to genus *Bacillus* sp. from different carbon sources (galactose, fructose, glucose, mannitol, sucrose, maltose and starch). *Bacillus* sp. 112A produced a maximum of 67.73% P(3HB) from glucose, 58.50% from sucrose, followed by 50.50% from starch as carbon substrates, but also produced the polymer from the others carbon sources but with a lower PHA yield. The authors also performed the polymer analysis.

3. Methods and Materials

3.1 Culture

The mixed microbial culture used in this work as a source of microorganisms was collected from the Sequencing Batch Reactor (SBR) under Aerobic Dynamic Feeding in order to select PHA-accumulating organisms from HSSL by Queirós (2012).

3.2 Culture Medium

3.2.1 HSSL pretreatment

The hardwood sulphite spent liquor (HSSL) from magnesium based acidic sulphite pulping of *Eucalyptus globulus* was supplied by *Caima – Indústria de Celulose SA* (Constância Portugal). Pre-evaporated HSSL was collected from inlet evaporator in a set of multiple-effect evaporators to avoid the presence of free SO₂ in liquor. The pretreatment of HSSL consisted in pH adjustment to 7.0 with 6M KOH followed by aeration with compressed air (2 h.L⁻¹). Afterwards, the liquor was centrifuged for 1 hour at 2000 rpm. The precipitated colloids were then filtered off using a 1 µm glass microfiber filter (Ahlstrom, grade 131) (Xavier et al. 2010).

3.2.2 Medium composition

The medium selected was the same used in the SBR for the selection of PHA-storing organism by Queirós 2012. The three carbon sources used were either the by-product HSSL (4.2 gCOD.L⁻¹) or one of the two major carbon sources present in HSSL, Sodium acetate (CH₃COONa) and Xylose (C₅H₁₀O₅) in concentrations of 0.172g.L⁻¹ and 0.360 g.L⁻¹, respectively. The composition in micronutrients of culture medium is listed in Table 5. Phosphate salts were prepared separately, in order to avoid precipitation with

magnesium salts during sterilization. The pH of the medium was kept above 7.0 before sterilization and for solid medium the amount of agar used was 20 g.L⁻¹. The solutions were then autoclaved and left to cool down in a laminar flow hood. After cooling, phosphates and carbon source were added to the medium and then it was distributed to petri dishes.

Table 5: Concentration of nutrients in the culture medium.

Compound	Concentration (g.L⁻¹)
Monopotassium phosphate (KH ₂ PO ₄)	0.016
Dipotassium phosphate (K ₂ HPO ₄)	0.064
Magnesium sulfate heptahydrate (MgSO ₄ ·7H ₂ O)	0.160
Calcium sulfate dihydrate (CaSO ₄ ·2H ₂ O)	0.080
Iron(III) chloride (FeCl ₃)	0.020
Sodium Molybdate dihydrate (Na ₂ MoO ₄ ·2H ₂ O)	0.008
Ammonium chloride (NH ₄ Cl)	0.160

3.3 Bacterial isolation

In order to isolate some PHA-accumulating bacteria from the selected MMC, solid culture medium was prepared (Table 5). A volume of 50 µL of the selected mixed culture were spread onto agar growth medium and the inoculated plates were therefore incubated at 20°C. Pure cultures of PHA-storing bacteria were isolated by repeated streaking of isolated colonies. The purity of the isolates was evaluated performing gram staining and FISH analysis. The cultures were then inoculated in liquid medium, 50 mL with the respective carbon source from where it was isolated, to determine the growth rate. Then, a 400 mL Erlenmeyer was inoculated with an inoculum from the previous liquid medium. Kinetic tests were performed in triplicates. Samples were taken to evaluate the culture growth (absorbance at 420 nm), substrate consumption and to perform Nile Blue staining.

3.4 Analytical Methods

3.4.1 Carbon Sources Analysis

Overall substrate consumption was determined using the Chemical Oxygen Demand (COD), through Spectroquant® photometric kit (Merck), according to the kit's instructions.

3.4.2 Biomass concentration

VSS (volatile suspended solids) were determined according to Standard Methods (American Public Health Association 2005). 50 mL of sample were filtered using a filter of 1 µm (Whatman). The filter was dried in the oven at 105°C for 2 hours, for the determination of the Total Suspended Solids (TSS). Afterwards, the same filter was placed in the muffle furnace at 505°C for 2 hours, for the VSS determination. The determination of biomass concentration was performed through the conversion of the absorbance at 420 nm (Abs_{420nm}) into concentration using a calibration curve of Abs_{420nm} versus biomass concentration, in VSS ($g.L^{-1}$).

3.5 Sample fixation and storage

Biomass was harvested by centrifugation and resuspended in 1 volume of 1x Phosphate Buffered Saline (PBS). This washing step was performed two more times. 1 volume of paraformaldehyde (4% (v/v)) was then added to the eppendorf with the resuspended biomass and afterwards incubated at 4°C for 2 hours. The fixed sample was centrifuged (5 min, 4°C, 12 000 rpm) and the supernatant poured away. Then the sample was resuspended in 1 volume of ice-cold 1xPBS and 1 volume of ice-cold 96% (v/v) ethanol was added. The sample was stored at -20°C (Amann et al. 1995).

3.6 Gram Staining

After sample fixation on slide, followed by air drying, the microscope slide was stained for 1 minute with crystal violet solution. Then the solution was removed with water. Further, the slide was treated with solution of lugol for 1 minute and the washed out with water, decolorized with acetone and dried. Lastly, the slide was covered with a solution of safranin for 1 minute and the washed with water. The slide was left to air dry and then it was observed under oil immersion at 1000x magnification with direct illumination resorting to Zeis Axioskop equipped with JVC TK-128OE Color Video Camera (VLC software), Figure 6 (Jenkins et al. 1986).

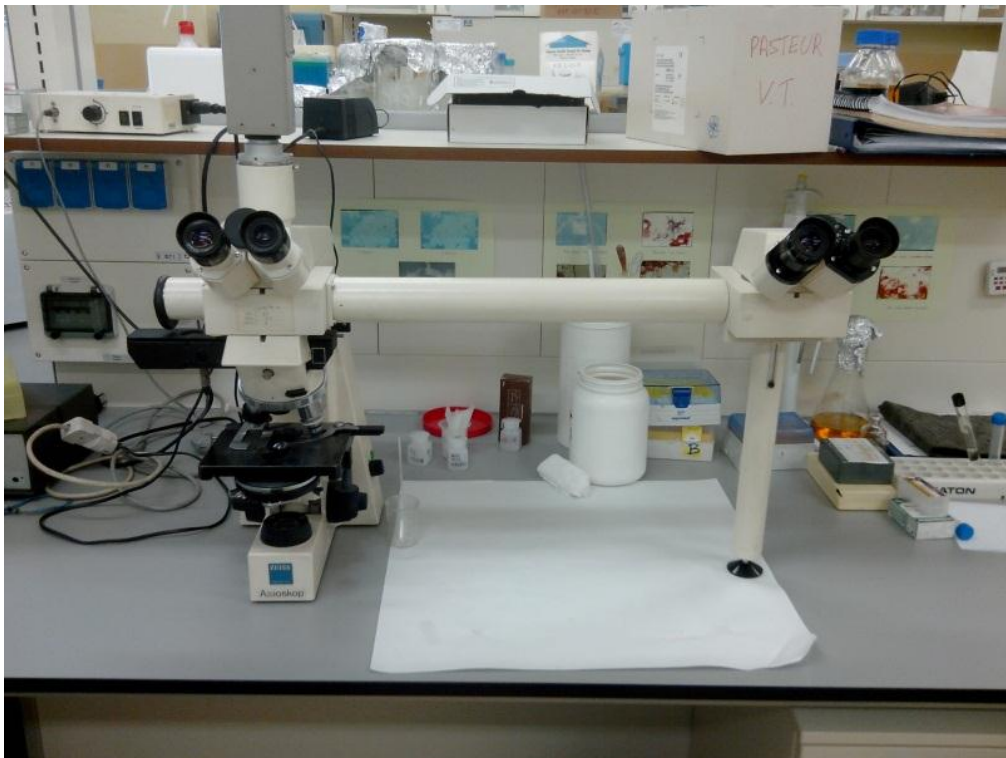


Figure 6: Optic microscope Zeis Axioskop equipped with JVC TK-128OE Color Video Camera.

3.7 Nile Blue Staining

Thin smears were prepared on microscope slides. After drying, the slides were submerged in a Nile blue solution (1% w/v), pre-heated at 55°C, for 10 minutes. The slides were recovered and washed with an 8% acetic acid solution for 1.5 minutes and left to air dry. Then slides were observed under oil immersion at 1000x magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 7 (Rees et al. 1992).



Figure 7: Epifluorescence microscope Olympus BX51, equipped with an Olympus XM10 camera

3.8 FISH analysis

10 μL of biomass fixed on formaldehyde (4 % v/v) were placed in individual wells of a Teflon coated slide and then air dried. The slide was then dehydrated in ethanol series of 50 %, 80% and 98%, 3 minutes each. Afterwards, 10 μL of hybridization buffer (previously prepared and according to stringency of the probes used) were added to each well of the slide and the remainder used to moisten a tissue paper for the hybridization chamber. Finally, 0.5 μL of probe (50 $\text{ng}\cdot\mu\text{L}^{-1}$) were added to the wells and the slide placed in the hybridization chamber at 46°C for 1.5 hours. Then, the slide was lightly washed with the pre-heated washing buffer and subsequently placed in a Falcon tube, containing the washing buffer at 48°C for 15 minutes. Cold Milli-Q water was used to remove the washing buffer from the slide. After air-drying, the slide was mounted with Vectorshield mounting medium containing DAPI stain (Amann et al. 1995).

The list of the probes used is shown in Table 6. The slide was observed under oil immersion at 1000x magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 7. Evaluation of probes efficiency was performed using the web tool mathFISH (Yilmaz et al. 2011).

Table 6: Probes and their sequences used in FISH.

Probe	Sequence (5' – 3')	Target	References
EUB338	GCTGCCTCCCGTAGGAGT	<i>Bacteria</i>	(Amann et al. 1995)
EUB338 II	GCAGCCACCCGTAGGTGT		
EUB338 III	GCTGCCACCCGTAGGTGT		
Alf968	GGTAAGGTTCTGCGCGTT	<i>Alphaproteobacteria</i> (except <i>Rickettsiales</i>)	(Neef 1997)
Bet42a	GCCTTCCCACCTTCGTTT	<i>Betaproteobacteria</i>	(Manz et al. 1992)
Gam42a	GCCTTCCCACATCGTTT	<i>Gammaproteobacteria</i>	(Manz et al. 1992)
Hgc69a	TATAGTTACCACCGCCGT	<i>Actinobacteria</i> (high GC Gram+ bacteria)	(Roller et al. 1994)
Sph120	GGGCAGATTCCCACGCGT	<i>Sphingomonadales</i>	(Eilers et al. 2000)

3.9 DNA extraction

For the DNA extraction of the selected mixed culture a bead-beating protocol was used (Rossetti et al., 2003). 2 ml sample were centrifuged and the pellet resuspended in 500 μ l 2x buffer A (200 mM Tris, pH 8.0, 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM CaCl₂) and 30 μ l lysozyme (100 mg/mL, freshly prepared) in a 2 ml screw-capped tube. The solution was incubated for 40 minutes at 37°C. A volume of 60 μ l proteinase K (20 mg.mL⁻¹) and 10 μ l 20% SDS were then added, and the solution was mixed by gentle inversion and incubated for a further 30 minutes at 50°C. The sample was mixed on a Mini-Beadbeater (Biospec Products), at the low setting for 2 minutes in the presence of 500 μ l phenol/chloroform/isoamyl alcohol (24:24:1), 200 μ l 20% SDS and approximately 0.3 g acid-washed zirconium beads (0.1 mm of diameter). After centrifuging at approximately 12 000 g at 4°C for 3 minutes to deposit the beads and sample debris, the lysate was extracted with 1 volume phenol/chloroform/isoamyl alcohol (24:24:1). The nucleic acids from the approximately 500 μ l aqueous phase were then precipitated by adding an equal volume of 2-propanol and 0.1 volume 3 M sodium acetate, pH 5.2, and incubating for 20 minutes on ice. The DNA pellet was recovered by centrifuging the solution at 12 000 g for 20 minutes at 4°C. The pellet was rinsed with 500 μ l 70% ice-cold ethanol and then, after discharging the ethanol solution by inverting the tube, air-dried and resuspended in 20 ml TE buffer (10 mM Tris/ HCl, 1 mM EDTA, pH 8.0) (Rossetti et al. 2003).

An extra step was performed to purify the extracted DNA by adding 20 μ L of phenol/chloroform/isoamyl alcohol (24:24:1) followed by mixing through gentle inversion and centrifugation at 13000 g for 15 minutes. To the supernatant obtained (around 20 μ L) were added 0.1 volume 3 M sodium acetate and 2 volumes of 70% ice-cold ethanol. The solution was kept on ice for 3 hours and the centrifuged at 13 000 g for 15 minutes. After discharging the ethanol solution by inverting the tube, the pellet was air-dried and resuspended in 100 μ l TE buffer (10 mM Tris/ HCl, 1 mM EDTA, pH 8.0).

3.10 PCR and Sequencing

This approach was applied to the isolates obtained, to DNA extracted from the MMC and to the clones of the 16S rDNA clone library. For this, a TaKaRa Ex Taq™ kit was used. The eppendorfs were prepared with general reaction mixture as described by the kit. The primers used were 27 forward (f) and 1492 reverse (r), for the isolates and genomic DNA of the MMC and T7f and U19r, which are specific plasmid primers, for the first screening of clones, and T7f was used with M13r, for the second screening Table 7. For the PCR the Perkin Elmer GeneAmp PCR System 2400 was used, programmed with two different cycle conditions according to the used primers.

When 27f and 1492r were employed, those were the following cycles:

- 94C for 4 minutes (1 cycle)
- 94°C for 1 minute, 48°C for 0,30 minute and 72°C for 2 minutes (35 cycles)
- 72°C for 12 minutes (1 Cycle).

When T7f and U19r were used, or T7f and M13r, the cycles were the following:

- 95C for 2 minutes (1 cycle)
- 95°C for 0.5 minute, 57°C for 1 minute and 72°C for 2 minutes (35 cycles)
- 72°C for 10 minutes (1 Cycle).

After the last cycle, the samples were cooled down until 4°C and an agarose (1%) electrophoresis was performed to check if there was amplification. Afterwards PCR products were purified using the QIAquick® PCR purification kit of (Quiagen, Milan, Italy) and quantified using the NanoDrop2000 Spectrophotometer (ThermoScientific, Milan, Italy).

The samples were then sent to BioFab (Rome, Italy) for sequencing, using the following primers: 530f, 926f, 907r and 519r, Table 7. After receiving the results, the sequences were assembled and run into BLAST.

Table 7: Probes and their sequences used in FISH.

	Primers	Sequence (5' – 3')
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
	T7f	TAATACGACTCACTATAGGG
	U19r	GTTTTCCCAGTCACGACGT
	M13r	TCACACAGGAAACAGCTATGAC
Sequencing	530f	GTGCCAGCMGCCGCGG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT
	519r	GWATTACCGCGGCKGCTG

M = C:A; Y = C:T; K = G:T; R = A:G; W = A:T; all 1:1

3.11 Cloning of the 16S rDNA

Two different cloning kits were used in order to create a 16S rDNA clone library. In the first screening performed, rDNAs were PCR-amplified from the extracted DNA from the microbial community as previously described, using the 530f and 1492r primers. After purification and quantification, the amplified rDNAs were ligated into pNZY28-A vector (NZYTech, Portugal), and transformed into NZYStar competent *E. coli*, according to the manufacturer instructions. The rDNA inserts from recombinant clones were amplified by PCR, using specific plasmid primers (U19 and T7) following the PCR protocol presented previously.

The second screening was performed as described above, however, using pGEM®-T Easy Vector and JM109 High Efficiency Competent Cells (Promega, USA). In addition to this, the primers used for the PCR were T7f and M13r.

4. Results and Discussion

4.1 Isolation attempts using HSSL – based medium

The isolation of diverse PHAs-producing bacteria may help to identify novel and more efficient PHAs producers, which may lead to better PHAs yield in a short period of time, thus cutting down production costs (Chien et al. 2007). Therefore, aiming the isolation of microorganisms able to store PHAs from the different carbon sources present in HSSL, from a mixed microbial culture selected under feast and famine conditions, isolation attempts by plating in solid medium containing HSSL were performed.

Despite the wide variety of PHAs-accumulating bacteria in different environments, only some reports have focused the potential for PHAs production of using resident bacterial species from activated sludge, which is generated in large quantities in biological wastewater treatment (Law et al. 2001; Reddy et al. 2008; Koller et al. 2011).

Biomass from the mixed microbial culture (MMC) previously selected under ADF conditions (Queirós 2012) was spread in solid medium containing HSSL and some colonies grew separately after 2 days. Five randomly chosen colonies were selected and continuously streaked onto solid medium in order to obtain pure cultures. The isolates were named as strains AF1 to AF5. The isolation of PHAs-accumulating bacteria from this sample of MMC had already been performed by Queirós (2012). The author isolated a bacteria identified as *Klebsiella* spp. using a plating isolation technique in a defined medium with xylose as carbon source.

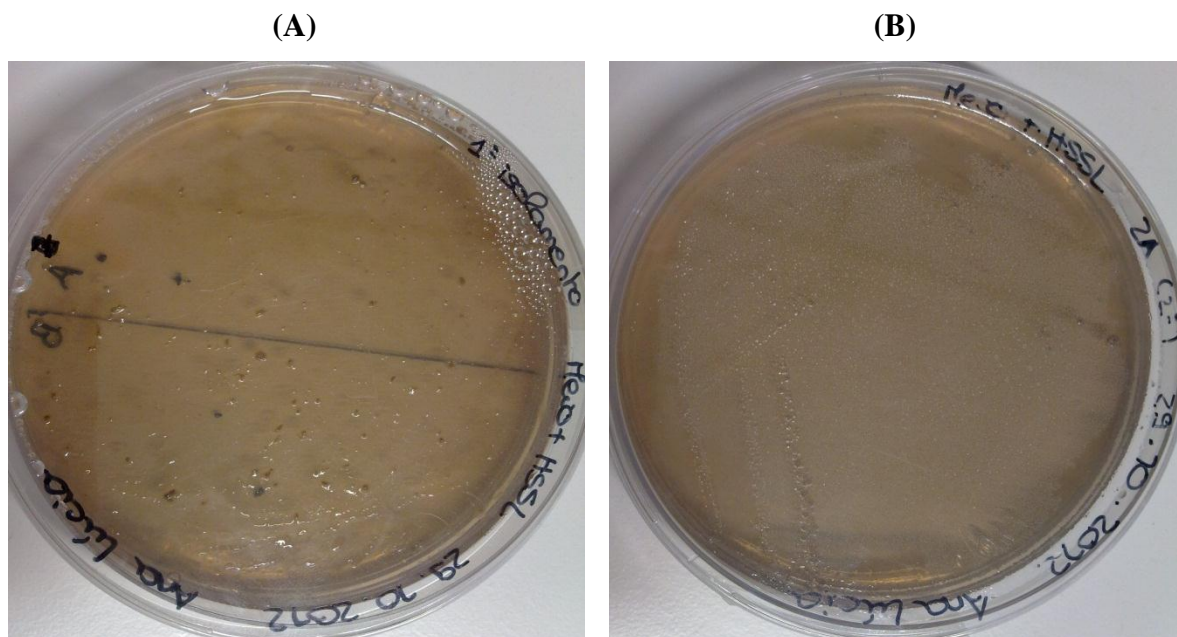


Figure 8: MMC plated in solid medium containing HSSL (A); isolated strain AF2 in pure culture, in solid medium containing HSSL (B).


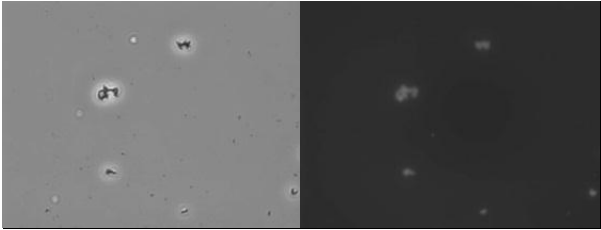

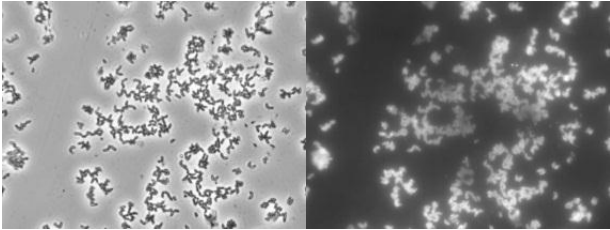
After several replating cycles, Gram and Nile Blue staining and FISH analysis were performed in order to evaluate the purity of the isolates, as well as to determine their morphology, PHAs-accumulating capability and elucidate on their taxonomy. The results are resumed in Table 8.


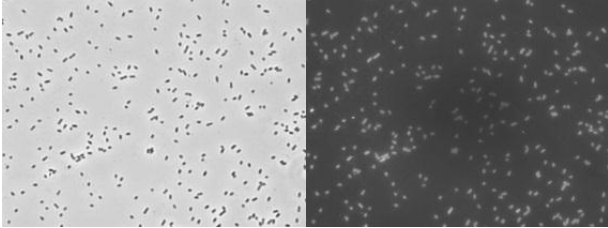
Gram staining showed that AF1 and AF2 were Gram variable, as they appeared to be Gram negative with Gram positive inclusions inside the cells, and the three remaining isolates were Gram negative. A higher number of Gram negative isolates was expected as it was the dominant morphotype on the MMC (Queirós 2012). Gram staining also allowed for the description of the morphology of the strains, which was confirmed by phase contrast microscopic observation. Strains AF1 and AF2 had a bacillus morphology, which was one of the main morphotype found on the MMC (Queirós 2012) and AF3, AF4 and AF5 a coccobacillus morphology, also detected in the MMC (Queirós 2012).

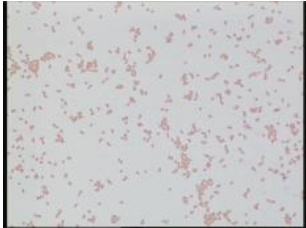
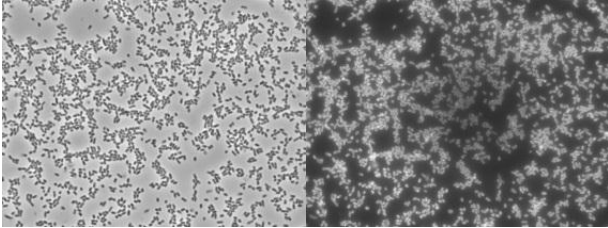
In order to verify the PHAs-accumulating abilities of the isolates, mineral base medium with sodium acetate at a concentration of 0.5g.L^{-1} was prepared and each one of the isolates was inoculated. Nile Blue staining, which is a specific staining for PHAs inclusions, allowed the confirmation that the five isolates were PHAs-accumulating bacteria. The presence of inclusion bodies of PHAs was easily observed by the presence of brilliant dots in the cells after Nile Blue staining procedure under epifluorescence microscopy. In the majority of cases the brilliant dots were replaced by the full fluorescence of the cells, which lead to believe that the cells stored a high amount of PHAs.


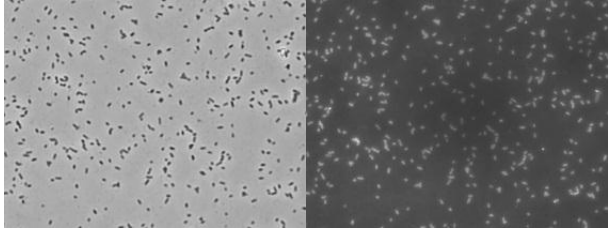
FISH analysis was performed as a preliminary taxonomic analysis of the isolates. From the five probes used (EUB338mix, Alf968, Bet42a, Gam42a and Hcg69a) the five isolates gave a positive result for EUB338mix, and therefore belonged to Bacteria Domain and three were positive for Gam42a, belonging to *Gammaproteobacteria*. The remaining two isolates showed negative result for Alf968, Bet42a, Gam42a and Hcg69a.

Table 8: Pictures of Gram and Nile Blue staining of the five isolates and the results of sequencing.

		Morphology		Nile Blue Stain (Phase Contrast / Epifluorescence)		Sequencing
	Morphology	Gram Stain				
AF1	<i>Bacilli</i>	Gram variable 		Complete Sequencing: 100% identity with <i>Rhodococcus</i> spp.		
AF2	<i>Bacilli</i>	Gram variable 		Partial Sequencing: 100% identity with <i>Rhodococcus</i> spp.		

AF3	<i>Coccobacilli</i>	Gram(-)		Complete Sequencing: 99% identity with <i>Pseudomonas</i> spp.
				

AF4	<i>Coccobacilli</i>	Gram(-)		Partial Sequencing: 100% identity with <i>Pseudomonas</i> spp.
				

AF5	<i>Coccobacilli</i>	Gram(-)		Partial Sequencing: 100% identity with <i>Pseudomonas</i> spp.
				

No bacteria belonging to *Alphaproteobacteria*, the dominant group present in the selected MMC (Queirós 2012), or to *Betaproteobacteria* were isolated. Therefore, as an attempt to isolate bacteria belonging to the dominant group, an isolation approach on solid medium containing acetic acid or xylose, as carbon source, was adopted. However, after obtaining three different isolates for each carbon source, FISH analysis was performed and the six isolates gave a positive result for *Gammaproteobacteria*. Therefore, the taxonomic affiliation of the isolates obtained in this study is consistent with the identity of the one isolated previously (Queirós 2012). They belonged either to *Gammaproteobacteria* or *Actinobacteria*. This finding meant that the culturable fraction of the MMC belonged to these two phylogenetic groups.

The isolation of bacteria belonging to *Gammaproteobacteria* instead of those belonging to the dominant classes has been previously observed (Gu et al. 2010). The isolation of nonylphenol ethoxylate-degrading bacteria from activated sludge had been assigned to *Gammaproteobacteria*, which was different from the results acquired using molecular techniques. Therefore, the authors used a new bacterial isolation strategy, by the use of gellan gum as a gelling reagent instead of agar. Gellan gum was used once it can effectively increase the cultivability of organisms and overcome some of the toxic effects that agar has on various groups of microorganisms (Gu et al. 2010). In addition, the clarity of the gellan gum gels allows to detect micro-colonies in plates that could not be seen on agar (Gu et al. 2010). The authors were able to isolate strains belonging to seven different genera including *Sphingobium* and *Achromobacter*, thus to *Alphaproteobacteria* and *Betaproteobacteria* classes (Gu et al. 2010). Future isolation attempts should be performed using this isolation strategy to verify if it would be possible to isolate bacteria belonging to the dominant group of the selected MMC.

4.1.1 Identification of isolates

The partial 16S rRNA sequencing followed by basic local alignment search tool (BLAST) analysis was performed in order to have a preliminary identification of the isolates. It showed that the isolated strains AF1 and AF2 had 100% similarity with *Rhodococcus* spp. and the remaining three, AF3, AF4 and AF5, had 100% similarity with

Pseudomonas spp. Taking these results into consideration, the complete sequencing of one of the isolated stains belonging to *Rhodococcus* spp. (AF1) and one to *Pseudomonas* spp. (AF3) was performed. The complete sequencing of the 16S rRNA allowed the identification of AF1 as *Rhodococcus* spp., which the preliminary affiliation was 100% to *Rhodococcus qingshengii* strain djl-6 (Xu et al. 2007). AF3 was identified as *Pseudomonas* spp, which the preliminary affiliation was 99% to *Pseudomonas libanensis* strain CIP 105460 (Dabboussi et al. 1999).

Members of genus *Rhodococcus* (*Actinobacteria* class) are widely distributed in natural environments, such as soils, water and marine sediments (Hernandez et al. 2008). These bacteria are well known for possessing a variety of catabolic pathways which allow them to degrade environmental pollutants and can be exploited to perform commercially useful bioconversions (de Carvalho et al. 2005). *Rhodococcus* species have been isolated from activated sludge samples, in which the main objective was the degradation of pollutants, such as estrogens and quinoline (Yoshimoto et al. 2004; Yu et al. 2007; Zhu et al. 2008). Moreover, degradation of phenolic compounds from *Rhodococcus* strains has also been reported in the literature (Martínková et al. 2009; Paisio et al. 2012). Lignosulphonates (LS) are the major component of HSSL, with a concentration around 78g.L⁻¹. LS include several phenolics, such as pyrogallol, syringic acid and gallic acid (Marques et al. 2009). Queirós (2012) reported consumption of LS during the PHAs production process. Therefore, it is possible that the *Rhodococcus* strains present in the MMC were one of the genera responsible for the consumption of these phenolics. Additionally, these microorganisms developed metabolic strategies to cope with these environments where nutrient limitation is common. One of these mechanisms may be the accumulation of storage compounds, such as PHAs, that can be utilized by cells as endogenous carbon sources and electron donors during periods of nutritional scarcity (Hernandez et al. 2008). A few members of this genus were described as PHAs-accumulators. *Rhodococcus ruber* NCIMB 40126 and *Rhodococcus* sp. ATCC 19070 were reported to accumulate PHAs copolymers containing 3HV units from a range of unrelated carbon sources, such as glucose and fructose (Haywood et al. 1991). Using glucose as carbon source a PHAs content of 14 and 21% of cell dry weight was achieved, respectively for each strain and using fructose the PHAs content was of 11 and 14% (Haywood et al. 1991). *Rhodococcus aetherivorans* IAR1 isolated from soil demonstrated ability to

produce 18 % cell dry weight of P(3HB-co-3HV) from toluene (Hori et al. 2009). Lastly, *R.jostii* RHA1 when cultivated with different carbon sources showed higher content of copolymer with gluconate (7.6% cell dry weight) (Hernandez et al. 2008).

The genus *Pseudomonas* belongs to class *Gammaproteobacteria*, family *Pseudomonadaceae* and includes a great variety of microorganisms that are ubiquitous in nature being found in water and soil, being characterized by an enormous biosynthetic capacity and versatility (Nitschke et al. 2011). Numerous *Pseudomonas* spp. strains were also reported as phenol-degrading bacteria (Agarry et al. 2008). Several *Pseudomonas* PHAs-producing strains have been isolated from diverse environments. *P. stutzeri* isolated from oil contaminated soil produced PHAs when grown on both soybean oil and glucose mineral media, producing PHAs up to 63 and 52% of cell dry weight, respectively (He et al. 1998). *P. extremaustralis* sp. 14-3^T isolated from a temporary pond in Antarctica was able to accumulate P(3HB) when octanoate was used as carbon source (López et al. 2009). *Pseudomonas* species have been mainly described as mcl-PHAs producers. However, a sludge isolated *P.aeruginosa* MTCC 7925 demonstrated good capacity to synthesize scl-lcl-PHAs copolymers from unrelated carbon sources containing scl-HAs (3HB and 3HV) and lcl-HAs of C₁₆ (3HHD) and C₁₈ (3HOD) units as the constituents (Singh et al. 2008). In activated sludge samples, the isolation and study of PHAs production of *Pseudomonas* spp strains was performed in some studies (Tsz-Chun et al. 2005; Porwal et al. 2008; Reddy et al. 2008; Kourmentza et al. 2009; Venkateswar Reddy et al. 2012a; Venkateswar Reddy et al. 2012b). Reddy et al. (2008) performed the isolation of PHAs producers from municipal sewage sludge, including activated sludge samples, and identified three *Pseudomonas* strains (Reddy et al. 2008). Porwal (2008) isolated 35 bacterial strains from diverse environmental sources, including activated sludge, and the activated sludge samples yielded 17 isolates, two of them identified as *P. stutzeri*, able to produce P(3HB) and hydrogen (Porwal et al. 2008). Considering studies of PHAs production by mixed cultures enriched under periodic feeding conditions in a SBR starting from activated sludge, the identification of *Pseudomonas* species as PHAs producers has also been performed (Dionisi et al. 2006; Lee et al. 2011). Lee et al. (2011) using nonanoic acid as substrate, identified *P.aeruginosa* as the dominant strain of the microbial community resorting to DGGE analysis and also performed the isolation of two strains, in which the 16S rDNA sequences revealed that they were closely related to *P.aeruginosa*. In the future,

further studies should include the design and validation of specific FISH probes for these two strains, in order to confirm their presence in the initial selected MMC.

4.2 Kinetic tests with isolates AF1 and AF3

After the isolation on agar growth medium, the isolated strains AF1 – *Rhodococcus* spp. and AF3 – *Pseudomonas* spp., were inoculated in liquid medium, consisting of mineral base containing HSSL or acetic acid or xylose. After the adaptation to the liquid medium, kinetic tests were performed, in order to characterize the two cultures. In Figures 9 and 10 are represented the growth and substrate consumption, in the three media, for the isolated strain AF1 and strain AF3, respectively. Kinetic tests were performed in triplicate. Therefore, the determinations regarding biomass (optical density and VSS) were performed in triplicate. However, COD was quantified for only one of the triplicates, due to technical limitations. The results of the kinetic parameters obtained are summarized in Table 9.

Table 9: Results obtained from the kinetic tests.

Isolate	Carbon Source	μ_{max} (h⁻¹)	t_d (h)	q_s (gS.gX⁻¹.h⁻¹)	$Y_{(X/S)}$ (gX.gS⁻¹)
AF1	HSSL	0.212 ± 0.0219	3.29 ± 0.344	0.466	0.454
	Acetic Acid	0.153 ± 0.0252	4.61 ± 0.800	0.519	0.295
	Xylose	0.188 ± 0.0238	3.73 ± 0.504	0.572	0.329
AF3	HSSL	0.251 ± 0.0526	2.84 ± 0.543	0.336	0.747
	Acetic Acid	0.194 ± 0.0147	3.58 ± 0.263	0.430	0.451
	Xylose	0.130 ± 0.00578	5.34 ± 0.239	0.264	0.492

4.2.1 Isolated strain AF1 – *Rhodococcus* spp.

The strain AF1 – *Rhodococcus* spp. showed high affinity with two media, the one with HSSL and with xylose, since it began to grow right after inoculation, and the lag

phase was undetectable. With acetic acid as sole carbon source, the strain presented a lag phase around two hours. As observed in Figure 9, a higher biomass concentration, of $0.510\pm 0.0966\text{gVSS.L}^{-1}$, was obtained in the assay using HSSL. This value is six times higher than the biomass concentration obtained using xylose as sole carbon source, $0.0867\pm 0.0538\text{gVSS.L}^{-1}$, and seventeen times higher than using acetic acid, $0.0308\pm 0.00247\text{gVSS.L}^{-1}$. One reason for these differences observed on biomass amount may be the lower concentration of substrate in two media with acetic acid and xylose, since the amount of carbon sources were determined in order to be approximately the same as the amount in which both carbon sources were present in the media with HSSL. However, as shown in Figure 9, this cannot be the only explanation, because xylose and acetic acid were still present at the end of the kinetic tests, acetic acid at much lower concentration $0.0467\text{ g COD.L}^{-1}$ than xylose $0.124\text{ g COD.L}^{-1}$, and cells were already in death phase. A possible explanation may be the lack of other components present in HSSL that are needed for microbial growth.

When grown in liquid medium with HSSL, the isolated strain AF1 presented a maximum specific growth rate (μ_{max}) of $0.212\pm 0.0219\text{ h}^{-1}$ corresponding to duplication time of $3.29\pm 0.344\text{ h}$. This μ_{max} was the highest showed by this strain. With acetic acid as sole carbon source AF1 presented a μ_{max} of $0.153\pm 0.0252\text{h}^{-1}$ corresponding to duplication time of $4.61\pm 0.800\text{h}$ and in xylose a μ_{max} of $0.188\pm 0.0238\text{h}^{-1}$ corresponding to duplication time of $3.73\pm 0.504\text{h}$. The growth and PHAs accumulation of *Rhodococcus* strains using acetate as carbon source as been reported, but the authors only report the PHAs accumulation content and monomers composition, not giving information towards the kinetic parameters of growth (Haywood et al. 1991; Hori et al. 2009). However, the effects of acetate concentrations on cell growth of a *Rhodococcus* strain, *R. rhodochrous* was previously shown (Honda et al. 1998). It was reported that cell growth was significantly inhibited at concentrations above 3g.L^{-1} and below 2.5 g.L^{-1} the specific growth rate was in a range of 0.15 to 0.16 h^{-1} (Honda et al. 1998), which goes according to our results. A very interesting finding was the fact that this strain was able to metabolize xylose, since there are no reports of utilization of this sugar from wild-type strains belonging to genus *Rhodococcus* (Hori et al. 2009; Xiong et al. 2012).

Isolation and characterization of PHAs-accumulating bacteria from HSSL

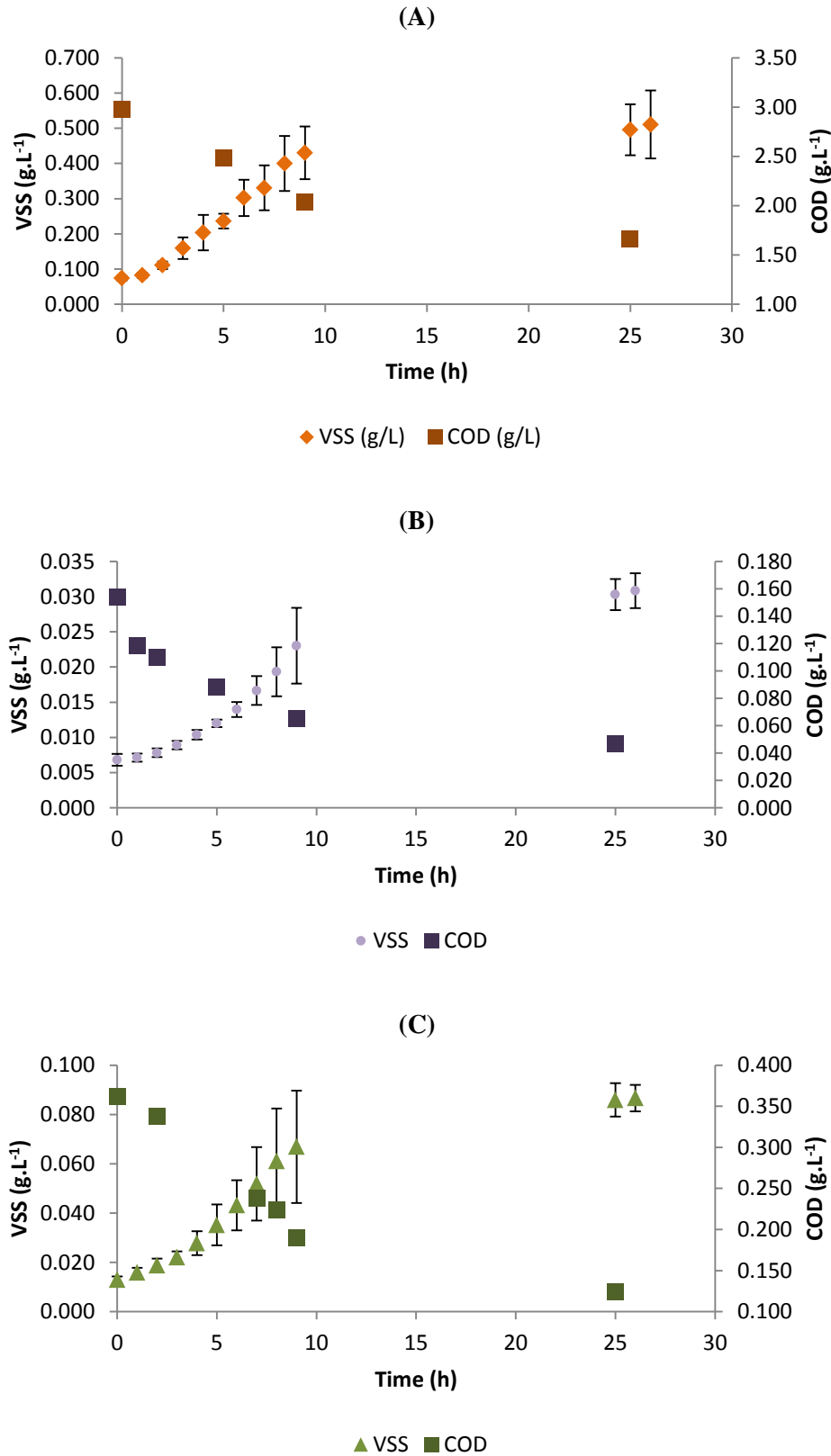


Figure 9: Microbial growth evolution, in gVSS.L^{-1} , and substrate consumption, in gCOD.L^{-1} , along the kinetic tests performed with the isolated strain AF1- *Rhodococcus* spp., in three different medium, each with a distinct carbon source: HSSL (C), acetic acid (B) and xylose (A).

4.2.2 Isolated strain AF3 – *Pseudomonas* spp.

The isolated strain AF3, identified as *Pseudomonas* spp., showed a very similar behavior as strain AF1. Both strain grew in the three media and resulted in higher biomass concentrations when grown in HSSL. However, the concentrations obtained were lower. In HSSL the biomass concentration obtained, of $0.174 \pm 0.0297 \text{gVSS.L}^{-1}$, was three times lower than for AF1 in the same medium. The biomass concentration achieved in HSSL was about three times higher than in the essays using acetic acid or xylose as sole carbon sources, which were respectively $0.0536 \pm 0.0121 \text{gVSS.L}^{-1}$ and $0.0642 \pm 0.0124 \text{gVSS.L}^{-1}$. Moreover, as can be observed in Table 9, the results showed the preference of both strains for growing in medium with HSSL, through the higher μ_{max} in the essays with HSSL, $0.212 \pm 0.0219 \text{h}^{-1}$ and $0.251 \pm 0.0526 \text{h}^{-1}$, presented by strains AF1 and AF3 respectively, as well as the biomass yield from substrate ($Y_{(X/S)}$), which were 0.454gX.gS^{-1} and 0.747gX.gS^{-1} , respectively. On the other hand, strain AF1 – *Rhodococcus* spp. showed a preference in consuming xylose and AF3 – *Pseudomonas* spp. for acetic acid, as they presented a higher specific substrate consumption rate (q_s) of $0.572 \text{gCOD.gVSS}^{-1}.\text{h}^{-1}$ and $0.430 \text{gCOD.gVSS}^{-1}.\text{h}^{-1}$, respectively, compared to those with the two other substrates. The growth and PHAs production has been previously reported for *Hydrogenophaga pseudoflava*, formerly called *Pseudomonas pseudoflava*, from the sugars present in hydrolysates from the hemicellulosic fraction of hardwood, as a carbon and energy source (Bertrand et al. 1990). In this study, the results obtained for xylose were similar to those of Bertrand et al (1990) in which *H. pseudoflava*, showed a μ_{max} of 0.130h^{-1} in the production of the copolymer from xylose.

The quantification of the components of HSSL along the time in the essays using HSSL is necessary in order to have a better understanding of the growth and consumption behavior of both strains using the by-product and should be performed in future studies. Queirós (2012) performed kinetic batch tests with the selected MMC and determined the kinetic parameters. In the study of the kinetics of PHAs production from HSSL components a μ_{max} of 0.143h^{-1} was obtained (Queirós 2012), which was lower than the obtained by the two isolated strains in this study. The same behavior was observed using

acetate or xylose as sole carbon sources, where the author obtained a μ_{\max} of 0.090 h^{-1} and 0.119 h^{-1} , respectively. The difference of behaviors concerning the two latter carbon sources may be partly explained due to the fact that the isolated strains AF1 and AF2 were first adapted to the liquid mediums before performing the kinetics tests, and in the case of the MMC the inoculum was directly removed from the SBR and inoculated into the new medium. Regarding the consumption of xylose, in the screening study performed by Lopes et al. (2009), the isolates showed a specific growth rate (μ_{\max}) range of $0.15\text{--}0.62 \text{ h}^{-1}$ using xylose as carbon source (Lopes et al. 2009). The results of this study are included in this range. Finally, taking into account that strains belonging to both isolated genera were previously reported as phenol-degrading bacteria (Agarry et al. 2008; Martínková et al. 2009; Paisio et al. 2012), in further studies, the possibility of both strains in consuming LS should be evaluated, through the quantification of LS on the growth curves in HSSL.

In PHAs production with the selected MMC from HSSL, acetic acid was completely consumed and some xylose remained in the medium (Queirós 2012). This allows for the use of the effluent of the PHAs production process to be used as substrate for other biological processes that use this pentose as carbon source, allowing the integration of another production process, under the Biorefinery concept. If the same behavior was verified in the production process using the isolates, for example, knowing that acetic acid acts as an inhibitor of many biological processes, its consumption would allow the subsequent utilization of the effluent for bioethanol production from xylose by *Scheffersonmycesstipiti stipitis* (Pereira et al. 2012).

Isolation and characterization of PHAs-accumulating bacteria from HSSL

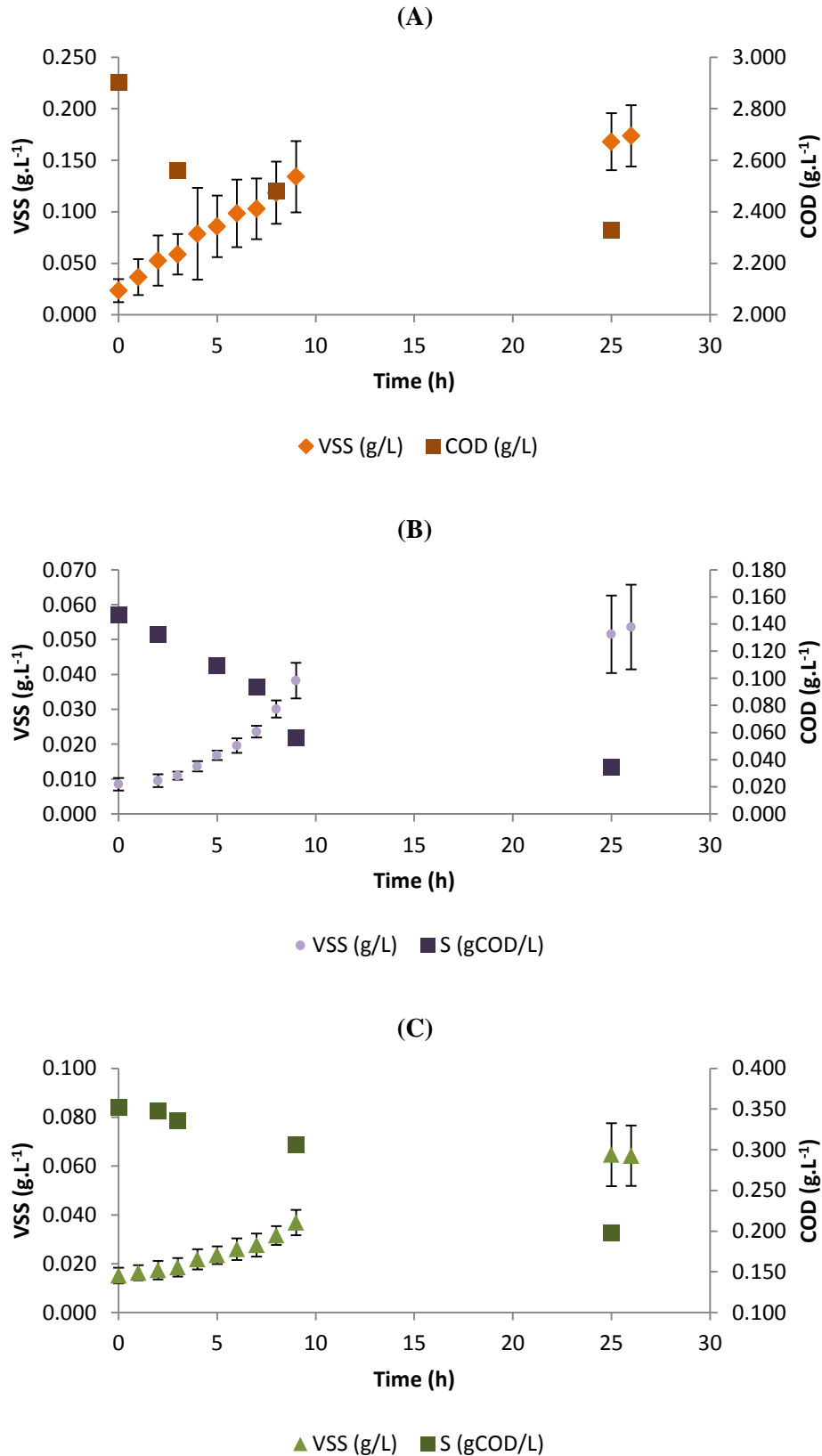


Figure 10: Microbial growth evolution, in gVSS.L^{-1} , and substrate consumption, in gCOD.L^{-1} , along the kinetic tests performed with the isolated strain AF3- *Pseudomonas* spp., in three different medium, each with a distinct carbon source: HSSL (A), acetic acid (B) and xylose (C).

4.2.2.1 PHAs accumulation

A qualitative evaluation of the PHAs-accumulating capability of both strains, AF1 – *Rhodococcus* spp. and AF3 – *Pseudomonas* spp., in liquid medium with the three different carbon sources analyzed (HSSL, acetic acid and xylose) was performed. Samples taken during the kinetics tests were observed under phase contrast and after Nile Blue staining. The results, as images of microscopic observation of AF1- *Rhodococcus* spp. and AF3 – *Pseudomonas* spp., are shown in Table 10 and Table 11, respectively. It was possible to observe that PHAs accumulation in bacterial cells increased along the incubation period reaching a maximum at the late exponential stage of the growth curve which corresponds to 9hours in Table 10 and Table 11. This PHAs accumulation behavior has been previously observed in other studies (Reddy et al. 2008; Queirós 2012). For both strains, in HSSL and xylose assays it was possible to clearly observe the PHAs inclusions inside cells. In acetic acid assay, cells were completely fluorescent, which meant that they were completely full of PHAs. The latter finding is somehow expected when acetic acid was the substrate, since it is a VFA that is more easily and rapidly consumed by microorganisms and accumulated as PHAs (Gumel et al. 2012). As the PHAs accumulation increased and reached the maximum PHAs content, some cells were completely fluorescent in the three tests. Nevertheless, the fluorescence intensity was always higher when acetic acid was the carbon source supplied. In addition, in the assays of PHAs production with HSSL, a higher intensity of fluorescence was clearly observed for strain AF1 than for strain AF3, indicating a higher content of PHAs produced by the former.

The quantification of PHAs accumulated and analysis of the monomers in each media should be determine in order to confirm that the inclusions correspond to PHAs and evaluate which polymer was accumulated, once both strains were reported in previous studies has having capacity to accumulate the copolymer P(3HB-co-3HV). Another consideration is the fact that it has been reported that some strains belonging to genus *Rhodococcus* accumulate triacylglycerols (TAGs). Moreover, the strains *Rhodococcus ruber* NCIMB 40126 and *Rhodococcus opacus* PD630 showed capability to accumulate both storage compounds simultaneously (PHAs and TAGs) (Alvarez et al. 2000;

Hernández et al. 2010). Nile Blue stain is specific for PHAs, not staining glycogen and polyphosphate, however can stain lipid bodies, therefore increasing the need to quantify and analyze samples using Gas Chromatography (GC). However due to some technical problems, that was not possible.

Lower PHAs yields have been previously obtained for pure cultures using hemicellulosic hydrolysates. This may be due to presence of residual toxins or inhibitors and and/or limitation of biologically available xylose monosaccharides in the fermentation medium (Keenan et al. 2006). Consequently, reinforcing the need to quantify PHAs along time and also evaluate the PHAs-accumulating capability of the isolates increasing the concentration of HSSL in the medium. It is important to note that in this study, PHAs productivity obtained was expected to be lower compared to other studies using pure cultures due to minor biomass and substrate concentrations in the system and the uncontrolled cultivation conditions, of pH and oxygen (Yan et al. 2008). Concentrations of biomass and substrate used in commercial production are significantly higher. For example, the cell concentration of *Alcaligenes latus* was 76 g.L⁻¹ and 5-20g.L⁻¹ of sucrose resulted in a productivity of 4.94 gPHB.L⁻¹.h⁻¹ (Wang et al. 1997).

An ideal organism for PHAs production would be a culture that could store high PHAs contents and grow rapidly on an inexpensive substrate (Yan et al. 2008). Several studies have shown that it was possible and potentially economical to develop PHAs production systems using activated sludge to isolate the PHAs-accumulating bacteria for PHAs production, and this study reinforces this. From both strains, AF1-Rhodococcus showed great potential for PHAs production from HSSL, due to its higher biomass concentration, μ_{\max} and fluorescence intensity after Nile Blue staining. Further studies are needed in order to characterize the polymers produced and to optimize the growth and PHAs accumulation.

Table 10: Images of Phase Contrast and Nile Blue staining of samples of AF1-*Rhodococcus spp.*, taken at different times of growth in liquid medium with three different carbon sources (HSSL, acetic acid and xylose)

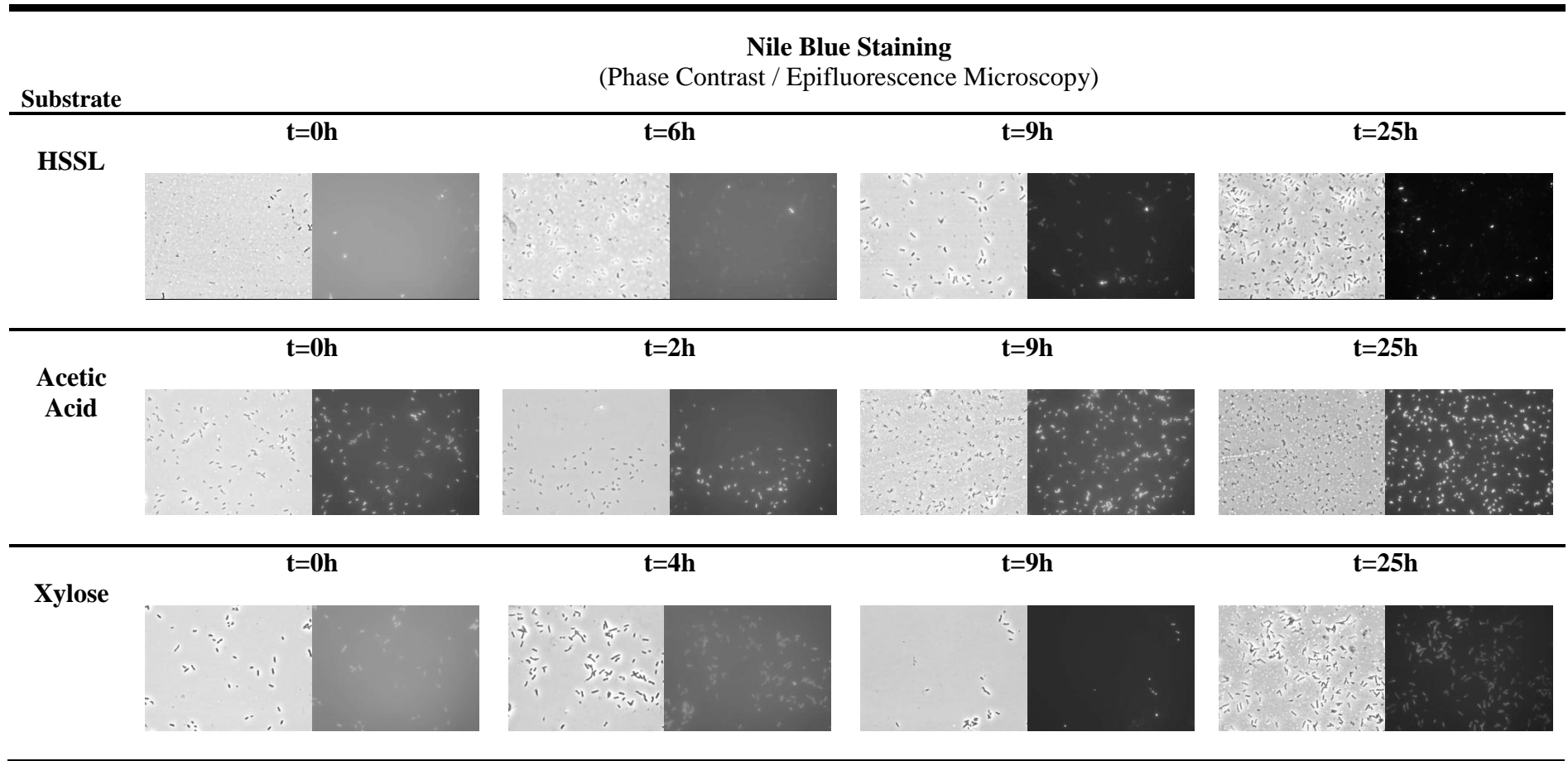
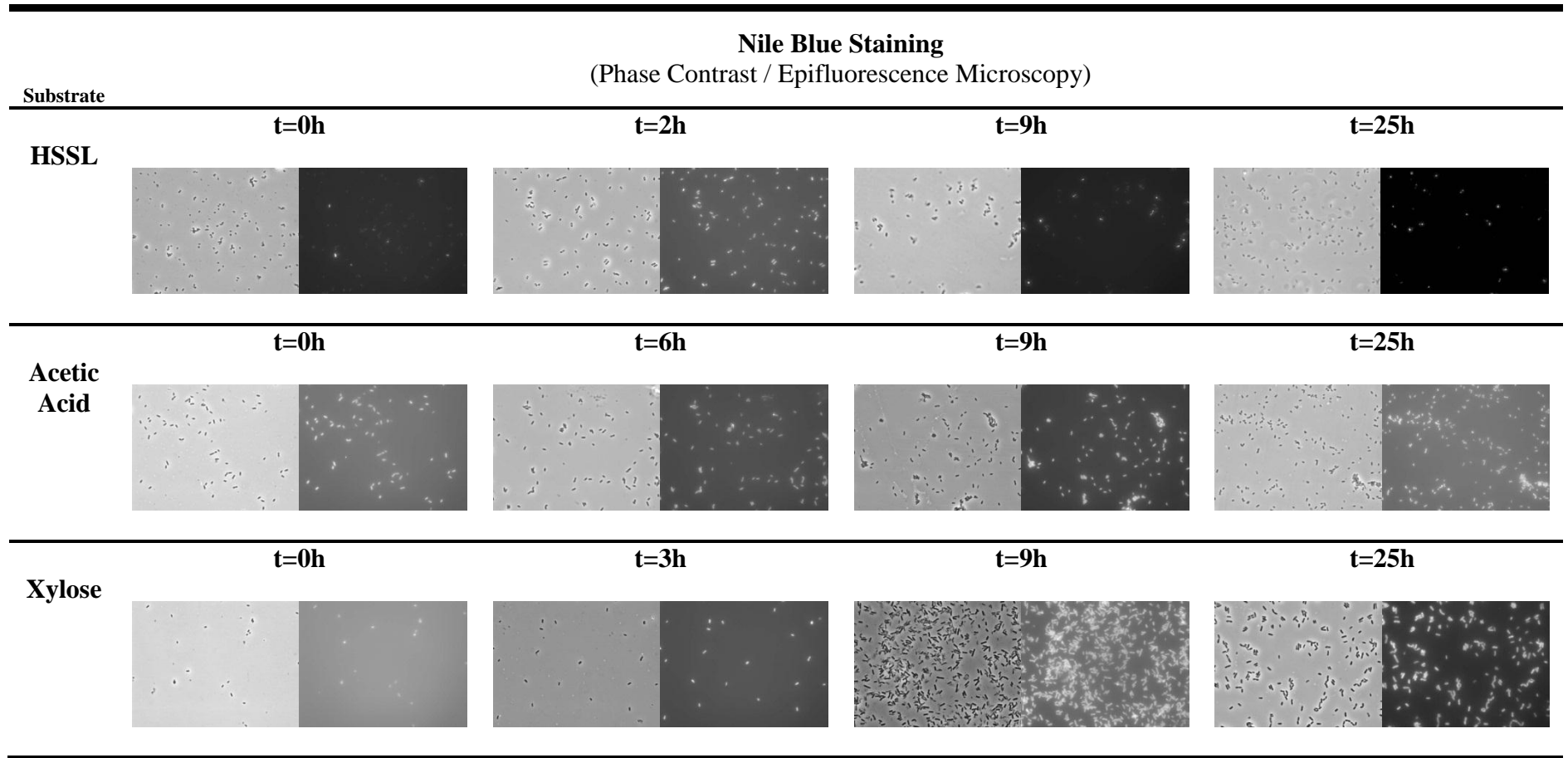


Table 11: Images of Phase Contrast and Nile Blue staining of samples of AF3-*Pseudomonas spp.*, taken at different times of growth in liquid medium with three different carbon sources (HSSL, acetic acid and xylose).



4.3 16S rDNA clone library

In order to identify some of the main bacteria responsible for PHAs accumulation of the MMC, a 16S rDNA clone library was constructed on DNA extracted from the selected MMC. The clones were identified through the complete 16S rRNA sequencing followed by basic local alignment search tool (BLAST) analysis. A total of 31 clones were obtained and 26 successfully identified. The clones were closely related with nine different genera: *Achromobacter* spp., *Comamonas* spp., *Clostridium* spp., *Methylobacillus* spp., *Novosphingobium* spp., *Pedobacter* spp., *Pleomorphomonas* spp., *Pseudomonas* spp. and *Sphingobium* spp. In Table 12 are presented the taxonomic affiliations of the clones obtained.

Table 12: Representation of the taxonomic affiliations of the clones obtained.

Taxonomic affiliation		Number of clones	Highest similarity	Identity	Accession number
<i>Achromobacter</i> spp.	<i>Betaproteobacteria</i>	5	<i>Achromobacter denitrificans</i> DSM 30026	99%	NR_042021.1
<i>Clostridium</i> spp.	<i>Firmicutes</i>	2	<i>Clostridium estertheticum</i> DSM 14864	98%	NR_042153.1
<i>Comamonas</i> spp.	<i>Betaproteobacteria</i>	3	<i>Comamonas testosteroni</i> CNB-2	99% *	NR_102841.1
<i>Methylobacillus</i> spp.	<i>Betaproteobacteria</i>	6	<i>Methylobacillus flagellatus</i> K	99% *	NR_074178.1
<i>Novosphingobium</i> spp.	<i>Alphaproteobacteria</i>	2	<i>Novosphingobium naphthalenivorans</i> TUT562	96%	NR_041046.1
<i>Pedobacter</i> spp.	<i>Bacteroidetes</i>	1	<i>Pedobacter terrae</i> DS-57	95%	NR_044005.1
<i>Pleomorphomonas</i> spp.	<i>Alphaproteobacteria</i>	2	<i>Pleomorphomonas koreensis</i> Y9	99% *	NR_040997.1
<i>Pseudomonas</i> spp.	<i>Gammaproteobacteria</i>	2	<i>Pseudomonas moraviensis</i> CCM 7280	99% *	NR_043314.1
<i>Sphingobium</i> spp.	<i>Alphaproteobacteria</i>	3	<i>Sphingobium</i> sp. SYK-6	95%	NR_074396.1

*partial sequencing

Nearly all the results were consistent with the findings of Queirós (2012) after characterized the selected MMC. *Alphaproteobacteria* was the dominant group of the MMC, accounting with 72.7 ± 4.0 %, followed by *Betaproteobacteria*, 11.1 ± 0.37 % and *Gammaproteobacteria*, 10.3 ± 0.3 %. A minor presence of *Deltaproteobacteria*, *Actinobacteria* and *Bacteroides* was also detected (Queirós 2012). The presence of *Pseudomonas* spp. in the MMC had already been verified, through its isolation in solid medium containing HHSL, as reported above. The presence of *Firmicutes* was not expected, once they had not been identified in the MMC through the FISH analysis (Queirós 2012). However, it is possible that they were present at a low amount and so undetectable.

It is important to notice that for *Novosphingobium* spp., *Sphingobium* spp. and *Pedobacter* spp., the identity values obtained in the BLAST analysis were relatively low. However, only for the two first microorganisms it is possible to say that these may be new strains, once the sequences of the microorganisms with highest identity are complete ones. Regarding the remaining strains, no conclusion may be established as only the partial sequencing was performed.

As can be observed in Table 13, species belonging to all the genera obtained from the clone library were previously identified or isolated from activated sludge samples, including *Pseudomonas* spp., which was discussed previously. They have been mostly been studied for their ability to grow in contaminated environments and to degrade those same contaminants. Only strains belonging to *Clostridium* spp., *Comamonas* spp., *Novosphingobium* spp., *Pleomorphomonas* spp. and *Sphingobium* spp. have been reported as PHAs-accumulating bacteria (Emeruwa et al. 1973; Thakor et al. 2005; Xie et al. 2005; Addison et al. 2007; Liang et al. 2010; Zakaria et al. 2010; Teeka et al. 2012).

Achromobacter spp. have been reported to be common microorganisms that use multiple organic pollutants in different environmental conditions (He et al. 2013). The ability of degrading several pollutants, such as pyridine, endosulfan, nonylphenoethoxylate and di-n-butyl phthalate, has been studied, including strains isolated from activated sludge (Li et al. 2009; Gu et al. 2010; Deng et al. 2011; He et al. 2013). This genus has been previously identified in selected activated sludge in an ADF reactor for PHAs accumulation. Dionisi et al. (2006) identified *Achromobacter* sp. after the construction of a clone library, however did not verify PHAs-producing capability (Dionisi et al. 2006). So, it is possible to conclude that this genus belongs to a fraction of the MMC that does not produce PHAs. In the same study, *Comamonas* sp. was also identified (Dionisi et al. 2006). However, the genus *Comamonas* has been evaluated for PHAs production. For example, *Comamonas testosteroni* showed ability to store mcl-PHAs when cultivated on vegetable oils (Thakor et al. 2005). More recently, *Comamonas* sp. EB172 was studied for its capability to produced PHAs from various carbon sources, including acetic acid (Zakaria et al. 2010).

Clostridium species have been mostly isolated from moderate environments but not only. *Clostridium estertheticum* DSM 14864 was isolated from a microbial mat located in the moated periphery of a perennial frozen lake (Spring et al. 2003). Its presence in activated sludge has also been detected (Mangayil et al. 2012). There are very few studies concerning the PHAs-accumulating capability of *Clostridium* species, however it is known that *Clostridium botulinum* accumulates P(3HB) during the growth phase and utilizes it as an energy source for sporulation (Emeruwa et al. 1973).

Three genera belonging to *Alphaproteobacteria* were identified, *Novosphingobium*, *Sphingobium* and *Pleomorphomonas*. The genus *Novosphingobium* includes a diverse group of bacteria displaying a number of unique traits that enable them to inhabit a variety of soil, sediment and aquatic environments (Addison et al. 2007). This genus was created to reclassify some species of the genus *Sphingomonas* by Takeuchi et al. (2001), in which the genus *Sphingomonas* was divided into four genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi et al. 2001). *Novosphingobium nitrogenifigens* Y88^T was the first species belonging to this genus reported as PHAs-accumulator. This strain was isolated from pulp and paper-mill of wastewaters undergoing biological treatment in a bioreactor operated under nitrogen-

limited conditions (Addison et al. 2007) and is capable to accumulate a high content of PHAs, over 80% of its biomass, as P(3HB), when grown on glucose (Smit et al. 2012). The genus *Sphingobium* is closely related to *Novosphingobium*. The strain *Sphingobium scionense* WP01^T was isolated from a polycyclic aromatic hydrocarbon (PAH) contaminated soil (Liang et al. 2010). This strains was able to produce PHAs, as P(3HB), accumulating up to 24.1% cell dry weight from glucose. Furthermore, *Sphingobium scionense* WP01^T also accumulated P(3HB) from PAH, in particular benzoate (36.4% of cell dry weight), naphthalene (15.7%) and biphenyl (13.3%) (Liang et al. 2011). *Pleomorphomonas* spp. are nitrogen-fixing bacteria and usually found associated to plants (Xie et al. 2005). Their presence in activated sludge samples has been previously detected (Duarte et al. 2010; Zheng et al. 2013). In particular, *P. oryzae* is able to use a wide variety of carbon sources, including xylose and acetate, and accumulate P(3HB) granules (Xie et al. 2005).

Isolation and characterization of PHAs-accumulating bacteria from HSSL

Table 13: Studies reporting the identification or isolation of strains belonging to the genera obtained in the clone library, from activated sludge samples as well as their PHAs production ability (if reported).

Studies of Identification / Isolation from Activated Sludge				PHAs-accumulating capability	
	Strain	Main Objective	Ref.	Strain	Ref.
<i>Achromobacter</i> spp.	<i>Achromobacter xylosoxidans</i> CS5	Biodegradation of endosulfan	(Li et al. 2009)		
	<i>Achromobacter</i> spp.	Isolation of nonylphenol ethoxylate-degrading bacteria	(Gu et al. 2010)		
	<i>Achromobacter</i> sp	Bioegradation of di-n-butyl phthalate	(He et al. 2013)		
<i>Clostridium</i> spp.	- <i>Clostridium sporogenes</i> CL3 - <i>Clostridium subterminale</i> DSM 758	Hydrogen production from glycerol	(Mangayil et al. 2012)	<i>Clostridium botulinum</i> ATCC 9564	(Emeruwa et al. 1973)
	<i>Clostridium</i> sp.	Study microbial community in printing and dyeing wastewater treatment system	(Yang et al. 2012)		
	<i>Clostridium uzonii</i>	Hydrogen production and phenol removal from palm oil mill effluent	(Mamimin et al. 2012)		
<i>Comamonas</i> spp.	<i>Comamonas badia</i>	Activated sludge system treating coking effluent	(Felföldi et al. 2010)	<i>Comamonas</i> sp. EB172	(Zakaria et al. 2010)
	<i>Comamonas</i> sp.	Assessment of denitrifying bacterial composition in activated sludge	(Srinandan et al. 2011)	<i>Comamonas testosteroni</i>	(Thakor et al. 2005)
	<i>Comamonas acidovorans</i>	Biodegration of phenol	(Safont et al. 2012)		

Table 13: Studies reporting the identification or isolation of strains belonging to the genera obtained in the clone library, from activated sludge samples as well as their PHAs production ability (if reported).

Studies of Identification / Isolation from Activated Sludge				PHAs-accumulating capability	
	Strain	Main Objective	Ref.	Strain	Ref.
<i>Methylobacillus</i> spp.	<i>Methylobacillus</i> sp.	Identification of Acetate- or Methanol-Assimilating Bacteria	(Osaka et al. 2006)		
	<i>Methylobacillus</i> sp.	antibiotics removal	(Xia et al. 2012)		
<i>Novosphingobium</i> spp.	<i>Novosphingobium tardaugs</i> ARI-1 ^T	Isolation of an β -estradiol-degrading bacterium	(Fujii et al. 2003)	<i>Novosphingobium nitrogenifigens</i> sp. Y88 ^T	(Addison et al. 2007)
	<i>Novosphingobium</i> sp. JEM-1	Estrogen removal	(Hashimoto et al. 2012)	<i>Novosphingobium</i> sp. THA_AIK7	(Teeka et al. 2012)
<i>Pedobacter</i> spp.	<i>Pedobacter</i> sp. EMB 36	Biodegradation of 2-chloro-nitrobenzene	(Xu et al. 2011)		
<i>Pleomorphomonas</i> spp.	<i>Pleomorphomonas</i> sp.	Treatment of linear alkylbenzene sulfonate	(Duarte et al. 2010)	<i>Pleomorphomonas oryzae</i>	(Xie et al. 2005)
	<i>Pleomorphomonas oryzae</i>	domestic wastewater treatment	(Zheng et al. 2013)		
<i>Sphingobium</i> spp.	<i>Sphingobium</i> sp. JZ-2	Bioegradation of pyrethroid	(Guo et al. 2009)	<i>Sphingobium scionense</i> sp. WP01 ^T	(Liang et al. 2010)
	<i>Sphingobium</i> spp.	Isolation of nonylphenol ethoxylate-degrading bacteria	(Gu et al. 2010)		

4.3.1.1 FISH analysis

In order to verify if strains *Novosphingobium* spp. and *Sphingobium* spp., obtained in the 16S rDNA clone library, were the dominant strains of the bacteria belonging to *Alphaproteobacteria* present in the MMC, FISH analysis was performed. Probe Sph120 was used, which is specific for the order *Shingomonadales*. Before FISH analysis, the specificity of the probe was studied using mathFISH tool (Yilmaz et al. 2011). Results are summarized in Table 15. It was possible to verify that Sph120 was not the best probe to be used in the case of *Novosphingobium* spp. despite the high value displayed for hybridization efficiency (0.9999) as it presented one mismatch very close to the middle of the probe sequence, and therefore decreasing the possibility of hybridization. Considering *Sphingobium* spp. it showed perfect alignment and high hybridization efficiency (0.8962).

However, the FISH analysis showed only few cells with positive result with the Sph120 probe. Consequently, the cells to which the probe hybridized must be the *Sphingobium* spp. present in the MMC. This allows concluding that *Sphingobium* spp. were not the dominant PHAs-accumulating bacteria present in the MMC.

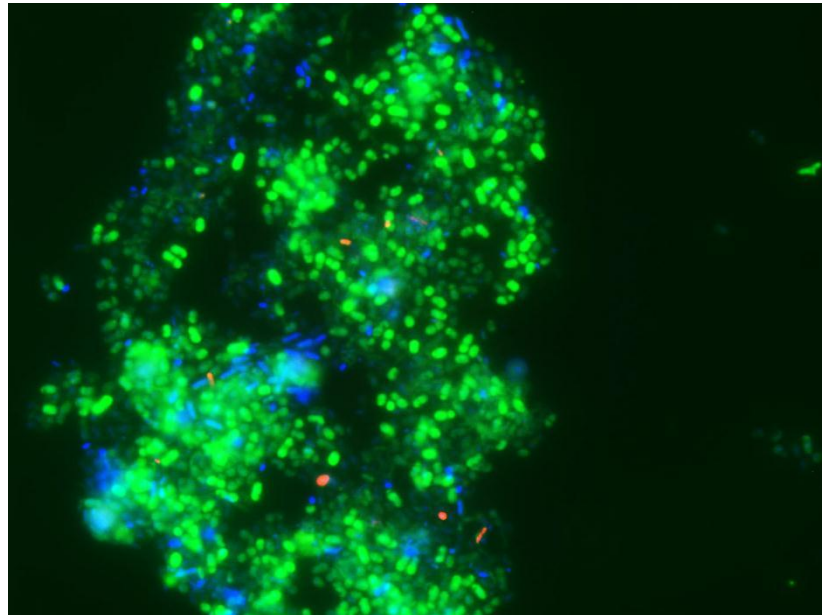


Figure 11: Overlap of FISH pictures. Green cells were only hybridized by EubMix probe and the red cells represent the cells hybridized by EubMix and Sph120 probes. Blue cells represent the remaining biomass that was not hybridized.

Further studies should consider the design and validation of probes specific for the genus *Novosphingobium*, in order to evaluate the presence in the MMC, determine precisely the abundance, and whether *Novosphingobium* spp. are the main responsables for PHAs production in the MMC. In addition to this, the FISH analysis of the remaining identified strains should be performed, including *Pleomorphomonas* spp., using both probes available or through the design and validation of specific probes.

5. Concluding remarks

Starting from a MMC selected under feast and famine conditions for PHAs production from HSSL, a by-product of pulp and paper industry, strains belonging to four out of the 6 groups identified of the microbial community were isolated or identified. Five strains were successfully isolated using an isolation approach using solid medium containing HSSL. Strains belonging to the dominant class of the MMC were identified, through the construction of a 16S rDNA clone library, constructed on DNA extracted from the selected MMC.

The five isolates obtained showed PHAs-accumulating capability. Two of the isolated strains were identified as *Rhodococcus* spp. with bacillus morphology. The remaining three had a coccobacillus morphology and were identified as *Pseudomonas* spp. The growth in liquid medium with HSSL or with the main carbon sources in HSSL (xylose or acetic acid), for one strain from each genus, AF1 – *Rhodococcus* spp. and AF3 – *Pseudomonas* spp., were further analyzed. Both strains grew and produced PHAs from each substrate, including AF1 – *Rhodococcus* spp. from xylose, which had not been previously reported. AF1 and AF2 showed preference for HSSL, and showed higher μ_{\max} , of $0.212 \pm 0.0219 \text{ h}^{-1}$ and $0.251 \pm 0.0526 \text{ h}^{-1}$, respectively, and greater biomass concentrations. Concerning the substrate consumption, AF1 showed a preference in consuming xylose and AF3 for acetic acid, as they presented a higher specific substrate consumption rate (q_s) of $0.572 \text{gCOD.gVSS}^{-1}.\text{h}^{-1}$ and $0.430 \text{gCOD.gVSS}^{-1}.\text{h}^{-1}$. Finally, from the qualitative evaluation of PHAs accumulation resulted the increase of inclusions along incubation time and a higher accumulation when acetic acid was used as sole carbon source.

Based on the information retrieved from the 16S rDNA clone library, *Novosphingobium* spp., *Sphingobium* spp. and *Pleomorphomonas* spp., belonging to the dominant class of the MMC, *Alphaproteobacteria* were highlighted. Furthermore, *Achromobacter* spp., *Clostridium* spp., *Comamonas* spp., *Methylobacillus* spp., *Pedobacter* spp. and *Pseudomonas* spp. were identified.

6. Future Prospects

In future works alternative isolation procedures need to be performed to isolate bacteria belonging to the dominant groups of the selected MMC. A possibility may concern isolation attempts performed using gellan gum as a gelling reagent instead of agar. In addition to this, specific FISH probes for all the strains identified by clone analysis, in order to confirm their presence in the initial selected MMC, should be designed and validated. The possibility of both the isolated strains, *Rhodococcus* spp. and *Pseudomonas* spp., in consuming LS should be evaluated, through the quantification of LS on the growth curves in HSSL. Finally, the quantification of PHAs accumulated and analysis of monomers composition in each media and determination of the properties of the polymers.

7. Bibliography

- Addison, S. L., Foote, S. M., Reid, N. M. and Lloyd-Jones, G. (2007). "*Novosphingobium nitrogenifigens* sp. nov., a polyhydroxyalkanoate-accumulating diazotroph isolated from a New Zealand pulp and paper wastewater." *International Journal of Systematic and Evolutionary Microbiology* **57**(11): 2467-2471.
- Agarry, S. E., Durojaiye, A. O. and Solomon, B. O. (2008). "*Microbial degradation of phenols: a review.*" *International Journal of Environment and Pollution* **32**(1): 12-28.
- Akar, A., Akkaya, E. U., Yesiladali, S. K., Celikyilmaz, G., Cokgor, E. U., Tamerler, C., Orhon, D. and Cakar, Z. P. (2006). "*Accumulation of polyhydroxyalkanoates by *Microlunatus phosphovor* under various growth conditions.*" *Journal of Industrial Microbiology and Biotechnology* **33**(3): 215-220.
- Akaraonye, E., Keshavarz, T. and Roy, I. (2010). "*Production of polyhydroxyalkanoates: the future green materials of choice.*" *Journal of Chemical Technology and Biotechnology* **85**(6): 732-743.
- Alvarez, H. M., Kalscheuer, R. and Steinbüchel, A. (2000). "*Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126.*" *Applied Microbiology and Biotechnology* **54**(2): 218-223.
- Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995). "*Phylogenetic identification and in situ detection of individual microbial cells without cultivation.*" *Microbiology Reviews* **59**(1): 143-169.
- American Public Health Association, A. W. W. A., Water Environment Federation (2005). *Standard methods for the examination of water and wastewater*. D.C., USA, American Public Health Association, Washington.
- Bertrand, J.-L., Ramsay, B. A., Ramsay, J. A. and Chavarie, C. (1990). "*Biosynthesis of Poly-p-Hydroxyalkanoates from Pentoses by *Pseudomonas pseudoflava*.*" *Applied And Environmental Microbiology* **56**(10): 3133-3138.
- Carreira, P., Mendes, J. A. S., Trovatti, E., Serafim, L. S., Freire, C. S. R., Silvestre, A. J. D. and Neto, C. P. (2011). "*Utilization of residues from agro-forest industries in the production of high value bacterial cellulose.*" *Bioresource Technology* **102**(15): 7354-7360.

- Castilho, L. R., Mitchel, D. A. and Freire, D. M. G. (2009). "Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation." *Bioresource Technology* **100**(23): 5996-6009.
- Chanprateep, S. (2010). "Current trends in biodegradable polyhydroxyalkanoates." *Journal of Bioscience and Bioengineering* **110**(6): 621-632.
- Chen, G. Q. (2009). "A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry." *Chemical Society Reviews* **38**(8): 2434-2446.
- Chen, G. Q. and Wu, Q. (2005). "The application of polyhydroxyalkanoates as tissue engineering materials." *Biomaterials* **26**(33): 6565-6578.
- Chen, Z., Li, Y., Wen, Q. and Zhang, H. (2011). "Biosynthesis of polyhydroxyalkanoate by *Gamma proteobacterium* WD-3 from volatile fatty acids." *Chemosphere* **82**(8): 1209-1213.
- Chien, C.-C., Chen, C.-C., Choi, M.-H., Kung, S.-S. and Wei, Y.-H. (2007). "Production of poly- β -hydroxybutyrate (PHB) by *Vibrio* spp. isolated from marine environment." *Journal of Biotechnology* **132**(3): 259-263.
- Dabboussi, F., Hamze, M., Elomari, M., Verhille, S., Baida, N., Izard, D. and Leclerc, H. (1999). "*Pseudomonas libanensis* sp. nov., a new species isolated from Lebanese spring waters." *International Journal of Systematic Bacteriology* **49 Pt 3**: 1091-1101.
- de Carvalho, C. C. and da Fonseca, M. M. (2005). "The remarkable *Rhodococcus erythropolis*." *Appl Microbiol Biotechnol* **67**(6): 715-726.
- Deng, X., Wei, C., Ren, Y. and Chai, X. (2011). "Isolation and Identification of *Achromobacter* sp. DN-06 and Evaluation of Its Pyridine Degradation Kinetics." *Water, Air, and Soil Pollution* **221**(1-4): 365-375.
- Dias, J. M. L., Lemos, P. C., Serafim, L. S., Oliveira, C., Eiroa, M., Albuquerque, M. G. E., Ramos, A. M., Oliveira, R. and Reis, M. A. M. (2006). "Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: From the substrate to the final product." *Macromolecular Bioscience* **6**(11): 885-906.
- Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S. and Beccari, M. (2006). "Effect of the applied organic load rate on biodegradable polymer production by mixed

- microbial cultures in a sequencing batch reactor.*" *Biotechnology and Bioengineering* **93**(1): 76-88.
- Duarte, I. C. S., Oliveira, L. L., Saavedra, N. K., Fantinatti-Garboggini, F., Menezes, C. B. A., Oliveira, V. M. and Varesche, M. B. A. (2010). "*Treatment of linear alkylbenzene sulfonate in a horizontal anaerobic immobilized biomass reactor.*" *Bioresource Technology* **101**(2): 606-612.
- Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000). "*Culturability and In Situ Abundance of Pelagic Bacteria from the North Sea.*" *Applied and Environmental Microbiology* **66**(7): 3044-3051.
- Emeruwa, A. C. and Hawirko, R. Z. (1973). "*Poly- β -Hydroxybutyrate Metabolism During Growth and Sporulation of Clostridium botulinum.*" *Journal of Bacteriology* **116**(2): 989-993.
- Felföldi, T., Székely, A. J., Gorál, R., Barkács, K., Scheirich, G., András, J., Rácz, A. and Márialigeti, K. (2010). "*Polyphasic bacterial community analysis of an aerobic activated sludge removing phenols and thiocyanate from coke plant effluent.*" *Bioresource Technology* **101**(10): 3406-3414.
- Fernandes, D. L. A., Pereira, S. R., Serafim, L. S., Evtuguin, D. V. and Xavier, A. M. R. B. (2012). *Second Generation Bioethanol from Lignocellulosics: Processing of Hardwood Sulphite Spent Liquor*. Croatia, InTech.
- Fujii, K., Satomi, M., Morita, N., Motomura, T., Tanaka, T. and Kikuchi, S. (2003). "*Novosphingobium tardaugens sp. nov., an oestradiol-degrading bacterium isolated from activated sludge of a sewage treatment plant in Tokyo.*" *International Journal of Systematic and Evolutionary Microbiology* **53**(1): 47-52.
- Gao, X., Chen, J. C., Wu, Q. and Chen, G. Q. (2011). "*Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels.*" *Current Opinion in Biotechnology* **22**(6): 768-774.
- Gomez, J. G. C., Méndez, B. S., Nikel, P. I., Pettinari, M. J., Prieto, M. A. and Silva, L. F. (2012). *Making Green Polymers Even Greener: Towards Sustainable Production of Polyhydroxyalkanoates from Agroindustrial By-Products*, InTech.
- Grage, K., Jahns, A. C., Parlane, N., Palanisamy, R., Rasiah, I. A., Atwood, J. A. and Rehm, B. H. A. (2009). "*Bacterial Polyhydroxyalkanoate Granules: Biogenesis,*

- Structure, and Potential Use as Nano-/Micro-Beads in Biotechnological and Biomedical Applications.*" *Biomacromolecules* **10**(4): 660-669.
- Gu, X., Zhang, Y., Zhang, J., Yang, M., Tamaki, H., Kamagata, Y. and Li, D. (2010). "Isolation of phylogenetically diverse nonylphenol ethoxylate-degrading bacteria and characterization of their corresponding biotransformation pathways." *Chemosphere* **80**(3): 216-222.
- Gumel, A. M., Annuar, M. S. M. and Chisti, Y. (2012). "Recent Advances in the Production, Recovery and Applications of Polyhydroxyalkanoates." *Journal of Polymers and the Environment*: 1-26.
- Guo, P., Wang, B., Hang, B., Li, L., Ali, S. W., He, J. and Li, S. (2009). "Pyrethroid-degrading *Sphingobium* sp. JZ-2 and the purification and characterization of a novel pyrethroid hydrolase." *International Biodeterioration and Biodegradation* **63**(8): 1107-1112.
- Hashimoto, T., Onda, K., Morita, T., Luxmy, B., Tada, K., Miya, A. and Murakami, T. (2012). "Contribution of the Estrogen-Degrading Bacterium *Novosphingobium* sp. Strain JEM-1 to Estrogen Removal in Wastewater Treatment." *Journal of Environmental Engineering* **136**(9): 890–896.
- Haywood, G. W., Anderson, A. J., Williams, D. R., Dawes, E. A. and Ewing, D. F. (1991). "Accumulation of a poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126." *International Journal of Biological Macromolecules* **13**(2): 83-88.
- Hazer, D. B., Kilicay, E. and Hazer, B. (2012). "Poly(3-hydroxyalkanoate)s: Diversification and biomedical applications A state of the art review." *Materials Science and Engineering C-Materials for Biological Applications* **32**(4): 637-647.
- He, W., Tian, W., Zhang, G., Chen, G.-Q. and Zhang, Z. (1998). "Production of novel polyhydroxyalkanoates by *Pseudomonas stutzeri* 1317 from glucose and soybean oil." *FEMS Microbiology Letters* **169**: 45-49.
- He, Z., Xiao, H., Tang, L., Min, H. and Lu, Z. (2013). "Biodegradation of di-n-butyl phthalate by a stable bacterial consortium, HD-1, enriched from activated sludge." *Bioresource Technology* **128**(0): 526-532.
- Hernández, M. A. and Alvarez, H. M. (2010). "Glycogen formation by *Rhodococcus* species and the effect of inhibition of lipid biosynthesis on glycogen

- accumulation in Rhodococcus opacus PD630.* FEMS Microbiology Letters **312**(1): 93-99.
- Hernandez, M. A., Mohn, W. W., Martinez, E., Rost, E., Alvarez, A. F. and Alvarez, H. M. (2008). "Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism." BMC Genomics **9**: 600.
- Honda, H., Sugiyama, H., Saito, I. and Kobayashi, T. (1998). "High cell density culture of *Rhodococcus rhodochrous* by pH-stat feeding and dibenzothiophene degradation." Journal of Fermentation and Bioengineering **85**(3): 334-338.
- Hori, K., Kobayashi, A., Ikeda, H. and Unno, H. (2009). "Rhodococcus aetherivorans IARI, a new bacterial strain synthesizing poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from toluene." Journal of Bioscience and Bioengineering **107**(2): 145-150.
- Jendrossek, D. (2009). "Polyhydroxyalkanoate Granules Are Complex Subcellular Organelles (Carbonosomes)." Journal of Bacteriology **191**(10): 3195-3202.
- Jenkins, D., Richard, M. G., Daigger, G. T., Agency, U. S. E. P. and Commission, S. A. W. R. (1986). *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*, Ridgeline Press.
- Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M. C. M. (2011). "Polyhydroxybutyrate Production From Lactate Using a Mixed Microbial Culture." Biotechnology and Bioengineering **108**(9): 2022-2035.
- Jiang, Y., Marang, L., Tamis, J., van Loosdrecht, M. C., Dijkman, H. and Kleerebezem, R. (2012). "Waste to resource: Converting paper mill wastewater to bioplastic." Water Research **46**(17): 5517-5530.
- Kamm, B., Gruber, P. R. and Kamm, M. (2006). *Biorefineries - Industrial Processes and Products: Status Quo and Future Directions*, WILEY-VCH Verlag GmbH.
- Keenan, T. M., Nakas, J. P. and Tanenbaum, S. W. (2006). "Polyhydroxyalkanoate copolymers from forest biomass." Journal of Industrial Microbiology and Biotechnology **33**(7): 616-626.
- Keshavarz, T. and Roy, I. (2010). "Polyhydroxyalkanoates: bioplastics with a green agenda." Current Opinion in Microbiology **13**(3): 321-326.

- Khanna, S. and Srivastava, A. K. (2005). "Recent advances in microbial polyhydroxyalkanoates." *Process Biochemistry* **40**(2): 607-619.
- Koller, M., Gasser, I., Schmid, F. and Berg, G. (2011). "Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms." *Engineering in Life Sciences* **11**(3): 222-237.
- Kourmentza, C., Ntaikou, I., Kornaros, M. and Lyberatos, G. (2009). "Production of PHAs from mixed and pure cultures of *Pseudomonas* sp. using short-chain fatty acids as carbon source under nitrogen limitation." *Desalination* **248**(1–3): 723-732.
- Law, K. H., Leung, Y. C., Lawford, H., Chua, H., Wai-Hung, L. and Yu, P. H. (2001). "Production of polyhydroxybutyrate by *Bacillus* species isolated from municipal activated sludge." *Applied Biochemistry and Biotechnology* **91-93**: 515-524.
- Lawford, H. G. and Rousseau, J. D. (1993). "Production of Ethanol from Pulp-Mill Hardwood and Softwood Spent Sulfite Liquors by Genetically-Engineered *Escherichia-Coli*." *Applied Biochemistry and Biotechnology* **39**: 667-685.
- Lee, S. H., Kim, J. H., Mishra, D., Ni, Y.-Y. and Rhee, Y. H. (2011). "Production of medium-chain-length polyhydroxyalkanoates by activated sludge enriched under periodic feeding with nonanoic acid." *Bioresource Technology* **102**: 6159–6166.
- Lee, S. Y. (1996). "Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria." *Trends in Biotechnology* **14**(11): 431-438.
- Lemoigne, M. (1926). "Produit de deshydratation et de polymerisation de l'acide b-oxybutyrique." *Bulletin de la Société de Chimie Biologique* **8**: 770-782.
- Lemos, P. C., Levantesi, C., Serafim, L. S., Rossetti, S., Reis, M. A. M. and Tandoi, V. (2008). "Microbial characterisation of polyhydroxyalkanoates storing populations selected under different operating conditions using a cell-sorting RT-PCR approach." *Applied Microbiology and Biotechnology* **78**(2): 351-360.
- Lemos, P. C., Serafim, L. S. and Reis, M. A. M. (2006). "Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding." *Journal of Biotechnology* **122**(2): 226-238.
- Li, W., Dai, Y., Xue, B., Li, Y., Peng, X., Zhang, J. and Yan, Y. (2009). "Biodegradation and detoxification of endosulfan in aqueous medium and soil by *Achromobacter xylosoxidans* strain CS5." *Journal of Hazardous Materials* **167**(1–3): 209-216.

- Liang, Q. and Lloyd-Jones, G. (2010). "*Sphingobium scionense* sp. nov., an aromatic hydrocarbon-degrading bacterium isolated from contaminated sawmill soil." *International Journal of Systematic and Evolutionary Microbiology* **60**(2): 413-416.
- Liang, Q. and Lloyd-Jones, G. (2011). Formation of Poly- β -hydroxybutyrate from Polycyclic Aromatic Hydrocarbons by *Sphingobium scionense* sp. WP01. Proceedings of the 2011 International Conference on Computer Distributed Control and Intelligent Environmental Monitoring, IEEE Computer Society: 2448-2451.
- Liu, K., Chua, H., Lo, W. H., Lawford, H. and Yu, P. H. (2002). "*Sphaerotilus natans* isolated from activated sludge and its production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)." *Applied Biochemistry and Biotechnology* **98-100**: 1061-1073.
- Liu, W. T., Mino, T., Matsuo, T. and Nakamura, K. (2000). "*Isolation, characterization and identification of polyhydroxyalkanoate-accumulating bacteria from activated sludge.*" *Journal of Bioscience and Bioengineering* **90**(5): 494-500.
- Lopes, M. S. G., Rocha, R. C. S., Zanotto, S. P., Gomez, J. G. C. and da Silva, L. F. (2009). "*Screening of bacteria to produce polyhydroxyalkanoates from xylose.*" *World Journal of Microbiology and Biotechnology* **25**(10): 1751-1756.
- López, N., Pettinari, M. J., Stackebrandt, E., Tribelli, P., Pötter, M., Steinbüchel, A. and Méndez, B. (2009). "*Pseudomonas extremaustralis* sp. nov., a Poly(3-hydroxybutyrate) Producer Isolated from an Antarctic Environment." *Current Microbiology* **59**(5): 514-519.
- Mamimin, C., Thongdumyu, P., Hniman, A., Prasertsan, P., Imai, T. and O-Thong, S. (2012). "*Simultaneous thermophilic hydrogen production and phenol removal from palm oil mill effluent by Thermoanaerobacterium-rich sludge.*" *International Journal of Hydrogen Energy* **37**(20): 15598-15606.
- Mangayil, R., Karp, M. and Santala, V. (2012). "*Bioconversion of crude glycerol from biodiesel production to hydrogen.*" *International Journal of Hydrogen Energy* **37**(17): 12198-12204.
- Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K. (1992). "*Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions.*" *Systematic and Applied Microbiology* **15**: 593 - 600.
- Marques, A. P., Evtuguin, D. V., Magina, S., Amado, F. M. L. and Prates, A. (2009). "*Chemical Composition of Spent Liquors from Acidic Magnesium-Based Sulphite*

- Pulping of Eucalyptus globulus.*" Journal of Wood Chemistry and Technology **29**(4): 322-336.
- Martínková, L., Uhnáková, B., Pátek, M., Nešvera, J. and Křen, V. (2009). "*Biodegradation potential of the genus Rhodococcus.*" Environment International **35**(1): 162-177.
- Moita, R. and Lemos, P. C. (2012). "*Biopolymers production from mixed cultures and pyrolysis by-products.*" Journal of Biotechnology **157**(4): 578-583.
- Mussatto, S. I. and Roberto, I. C. (2004). "*Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review.*" Bioresource Technology **93**(1): 1-10.
- Neef, A. (1997). *Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen.* PhD Thesis, Technische Universität München.
- Nitschke, M., Costa, S. G. V. A. O. and Contiero, J. (2011). "*Rhamnolipids and PHAs: Recent reports on Pseudomonas-derived molecules of increasing industrial interest.*" Process Biochemistry **46**(3): 621-630.
- NREL. (2012). "National Renewable Energy Laboratory - What Is a Biorefinery?" Retrieved 22.10.2012, from <http://www.nrel.gov/biomass/biorefinery.html>
- Octave, S. and Thomas, D. (2009). "*Biorefinery: Toward an industrial metabolism.*" Biochimie **91**(6): 659-664.
- Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N. and Inamori, Y. (2006). "*Identification of Acetate- or Methanol-Assimilating Bacteria under Nitrate-Reducing Conditions by Stable-Isotope Probing.*" Microbial Ecology **52**(2): 253-266.
- Paisio, C., Talano, M., González, P., Busto, V., Talou, J. and Agostini, E. (2012). "*Isolation and characterization of a Rhodococcus strain with phenol-degrading ability and its potential use for tannery effluent biotreatment.*" Environmental Science and Pollution Research **19**(8): 3430-3439.
- Palmqvist, E. and Hahn-Hagerdal, B. (2000). "*Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.*" Bioresource Technology **74**(1): 25-33.

- Pei, L., Schmidt, M. and Wei, W. (2011). *Conversion of Biomass into Bioplastics and Their Potential Environmental Impacts*, InTech.
- Pereira, S. R., Ivanusa, S., Evtuguin, D. V., Serafim, L. S. and Xavier, A. M. R. B. (2012). "Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol." *Bioresource Technology* **103**(1): 131-135.
- Philip, S., Keshavarz, T. and Roy, I. (2007). "Polyhydroxyalkanoates: biodegradable polymers with a range of applications." *Journal of Chemical Technology and Biotechnology* **82**(3): 233-247.
- Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., Purohit, H. J., Sharma, R., Singh Patel, S. K. and Kalia, V. C. (2008). "Hydrogen and polyhydroxybutyrate producing abilities of microbes from diverse habitats by dark fermentative process." *Bioresource Technology* **99**(13): 5444-5451.
- Queirós, D. C. (2012). *PHA production by mixed cultures: a way to valorize industrial waste*. Chemistry Department. Aveiro. MSc. Thesis, University of Aveiro.
- Reddy, C. S. K., Ghai, R., Rashmi and Kalia, V. C. (2003). "Polyhydroxyalkanoates: an overview." *Bioresource Technology* **87**(2): 137-146.
- Reddy, S. V., Thirumala, M., Reddy, T. V. K. and Mahmood, S. K. (2008). "Isolation of bacteria producing polyhydroxyalkanoates (PHA) from municipal sewage sludge." *World Journal of Microbiology and Biotechnology* **24**(12): 2949-2955.
- Rees, G. N., Vasiliadis, G., May, J. W. and Bayly, R. C. (1992). "Differentiation of Polyphosphate and Poly-Beta-Hydroxybutyrate Granules in an *Acinetobacter Sp* Isolated from Activated-Sludge." *FEMS Microbiology Letters* **94**(1-2): 171-174.
- Rehm, B. H. A. (2010). "Bacterial polymers: biosynthesis, modifications and applications." *Nature Reviews Microbiology* **8**(8): 578-592.
- Roller, C., Wagner, M., Amann, R., Ludwig, W. and Schleifer, K. (1994). "In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA- targeted oligonucleotides." *Microbiology* **140**: 2849-2858.
- Rossetti, S., Blackall, L. L., Majone, M., Hugenholtz, P., Plumb, J. J. and Tandoi, V. (2003). "Kinetic and phylogenetic characterization of an anaerobic dechlorinating microbial community." *Microbiology* **149**(Pt 2): 459-469.

- Safont, B., Vitas, A. I. and Peñas, F. J. (2012). "*Isolation and characterization of phenol degrading bacteria immobilized onto cyclodextrin-hydrogel particles within a draft tube spouted bed bioreactor.*" *Biochemical Engineering Journal* **64**(0): 69-75.
- Salehizadeh, H. and Van Loosdrecht, M. C. M. (2004). "*Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance.*" *Biotechnology Advances* **22**(3): 261-279.
- Sangkharak, K. and Prasertsan, P. (2012). "*Screening and identification of polyhydroxyalkanoates producing bacteria and biochemical characterization of their possible application.*" *Journal of General and Applied Microbiology* **58**(3): 173-182.
- Serafim, L. S., Lemos, P. C., Albuquerque, M. G. E. and Reis, M. A. M. (2008). "*Strategies for PHA production by mixed cultures and renewable waste materials.*" *Applied Microbiology and Biotechnology* **81**(4): 615-628.
- Serafim, L. S., Lemos, P. C., Rossetti, S., Levantesi, C., Tandoi, V. and Reis, M. A. (2006). "*Microbial community analysis with a high PHA storage capacity.*" *Water Science and Technology* **54**(1): 183-188.
- Singh, A. K. and Mallick, N. (2008). "*Enhanced production of SCL-LCL-PHA co-polymer by sludge-isolated Pseudomonas aeruginosa MTCC 7925.*" *Letters in Applied Microbiology* **46**(3): 350-357.
- Smit, A. M., Strabala, T. J., Peng, L., Rawson, P., Lloyd-Jones, G. and Jordan, T. W. (2012). "*Proteomic phenotyping of Novosphingobium nitrogenifigens reveals a robust capacity for simultaneous nitrogen fixation, polyhydroxyalkanoate production, and resistance to reactive oxygen species.*" *Appl Environ Microbiol* **78**(14): 4802-4815.
- Spring, S., Merkhoffer, B., Weiss, N., Kroppenstedt, R. M., Hippe, H. and Stackebrandt, E. (2003). "*Characterization of novel psychrophilic clostridia from an Antarctic microbial mat: description of Clostridium frigoris sp. nov., Clostridium lacusfryxellense sp. nov., Clostridium bowmanii sp. nov. and Clostridium psychrophilum sp. nov. and reclassification of Clostridium laramiense as Clostridium estertheticum subsp. laramiense subsp. nov.*" *International Journal of Systematic and Evolutionary Microbiology* **53**(4): 1019-1029.

- Srinandan, C. S., Shah, M., Patel, B. and Nerurkar, A. S. (2011). "Assessment of denitrifying bacterial composition in activated sludge." *Bioresource Technology* **102**(20): 9481-9489.
- Sudesh, K., Abe, H. and Doi, Y. (2000). "Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters." *Progress in Polymer Science* **25**(10): 1503-1555.
- Takeuchi, M., Hamana, K. and Hiraishi, A. (2001). "Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses." *International Journal of Systematic and Evolutionary Microbiology* **51**(4): 1405-1417.
- Teeka, J., Imai, T., Reungsang, A., Cheng, X., Yuliani, E., Thiantanankul, J., Poomipuk, N., Yamaguchi, J., Jeenanong, A., Higuchi, T., Yamamoto, K. and Sekine, M. (2012). "Characterization of polyhydroxyalkanoates (PHAs) biosynthesis by isolated *Novosphingobium* sp. *THA_AIK7* using crude glycerol." *Journal of Industrial Microbiology and Biotechnology* **39**(5): 749-758.
- Thakor, N., Trivedi, U. and Patel, K. C. (2005). "Biosynthesis of medium chain length poly(3-hydroxyalkanoates) (mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils." *Bioresource Technology* **96**(17): 1843-1850.
- Thirumala, M., Reddy, S. V. and Mahmood, S. K. (2010). "Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge." *Journal of Industrial Microbiology and Biotechnology* **37**(3): 271-278.
- Tsz-Chun, M., Chan, P. L., Lawford, H., Chua, H., Lo, W. H. and Yu, P. H. (2005). "Microbial synthesis and characterization of physicochemical properties of polyhydroxyalkanoates (PHAs) produced by bacteria isolated from activated sludge obtained from the municipal wastewater works in Hong Kong." *Applied Biochemistry and Biotechnology* **122**(1-3): 731-739.
- Venkateswar Reddy, M., Nikhil, G. N., Venkata Mohan, S., Swamy, Y. V. and Sarma, P. N. (2012b). "*Pseudomonas otitidis* as a potential biocatalyst for polyhydroxyalkanoates (PHA) synthesis using synthetic wastewater and acidogenic effluents." *Bioresource Technology* **123**(0): 471-479.

- Venkateswar Reddy, M. and Venkata Mohan, S. (2012a). "Effect of substrate load and nutrients concentration on the polyhydroxyalkanoates (PHA) production using mixed consortia through wastewater treatment." *Bioresource Technology* **114**(0): 573-582.
- Villano, M., Beccari, M., Dionisi, D., Lampis, S., Miccheli, A., Vallini, G. and Majone, M. (2010). "Effect of pH on the production of bacterial polyhydroxyalkanoates by mixed cultures enriched under periodic feeding." *Process Biochemistry* **45**(5): 714-723.
- Wang, F. and Lee, S. Y. (1997). "Poly(3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of *Alcaligenes latus* under Nitrogen Limitation." *Applied and Environmental Microbiology* **63**(9): 3703-3706.
- Wong, P. A. L., Chua, H., Lo, W. H., Lawford, H. G. and Yu, P. H. (2002). "Production of specific copolymers of polyhydroxyalkanoates from industrial waste." *Applied Biochemistry and Biotechnology* **98**: 655-662.
- Xavier, A. M. R. B., Correia, M. F., Pereira, S. R. and Evtuguin, D. V. (2010). "Second-generation bioethanol from eucalypt sulphite spent liquor." *Bioresource Technology* **101**(8): 2755-2761.
- Xia, S., Jia, R., Feng, F., Xie, K., Li, H., Jing, D. and Xu, X. (2012). "Effect of solids retention time on antibiotics removal performance and microbial communities in an A/O-MBR process." *Bioresource Technology* **106**(0): 36-43.
- Xie, C.-H. and Yokota, A. (2005). "*Pleomorphomonas oryzae* gen. nov., sp. nov., a nitrogen-fixing bacterium isolated from paddy soil of *Oryza sativa*." *Int J Syst Evol Microbiol* **55**(3): 1233-1237.
- Xiong, X., Wang, X. and Chen, S. (2012). "Engineering of a Xylose Metabolic Pathway in *Rhodococcus* Strains." *Applied and Environmental Microbiology*.
- Xu, J.-L., He, J., Wang, Z.-C., Wang, K., Li, W.-J., Tang, S.-K. and Li, S.-P. (2007). "*Rhodococcus qingshengii* sp. nov., a carbendazim-degrading bacterium." *International Journal of Systematic and Evolutionary Microbiology* **57**(12): 2754-2757.
- Xu, X., Lin, H., Zhu, L., Yang, Y. and Feng, J. (2011). "Enhanced biodegradation of 2-chloronitro-benzene using a coupled zero-valent iron column and sequencing batch

- reactor system." *Journal of Chemical Technology and Biotechnology* **86**(7): 993-1000.
- Yan, S., Subramanian, S. B., Tyagi, R. D. and Surampalli, R. Y. (2008). "*Polymer production by bacterial strains isolated from activated sludge treating municipal wastewater.*" *Water Science and Technology* **57**(4): 533-539.
- Yang, Q., Wang, J., Wang, H., Chen, X., Ren, S., Li, X., Xu, Y., Zhang, H. and Li, X. (2012). "*Evolution of the microbial community in a full-scale printing and dyeing wastewater treatment system.*" *Bioresource Technology* **117**(0): 155-163.
- Yilmaz, L. S., Parkernar, S., Noguera, D. R. and (2011). "*mathFISH, a Web Tool That Uses Thermodynamics-Based Mathematical Models for In Silico Evaluation of Oligonucleotide Probes for Fluorescence In Situ Hybridization.*" *Applied and Environmental Microbiology* **77**: 1118-1122.
- Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K., Saino, H., Sawada, H. and Omura, H. (2004). "*Degradation of estrogens by Rhodococcus zopfii and Rhodococcus equi isolates from activated sludge in wastewater treatment plants.*" *Applied and Environmental Microbiology* **70**(9): 5283-5289.
- Yu, C. P., Roh, H. and Chu, K. H. (2007). "*17beta-estradiol-degrading bacteria isolated from activated sludge.*" *Environmental Science and Technology* **41**(2): 486-492.
- Zakaria, M. R., Ariffin, H., Mohd Johar, N. A., Abd-Aziz, S., Nishida, H., Shirai, Y. and Hassan, M. A. (2010). "*Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer from wild-type Comamonas sp. EB172.*" *Polymer Degradation and Stability* **95**(8): 1382-1386.
- Zheng, S., Cui, C., Quan, Y. and Sun, J. (2013). "*Microaerobic DO-induced microbial mechanisms responsible for enormous energy saving in upflow microaerobic sludge blanket reactor.*" *Bioresource Technology* **140**(0): 192-198.
- Zhu, S. N., Liu, D. Q., Fan, L. and Ni, J. R. (2008). "*Degradation of quinoline by Rhodococcus sp. QL2 isolated from activated sludge.*" *Journal of Hazardous Materials* **160**(2-3): 289-294.
- Zinn, M., Witholt, B. and Egli, T. (2001). "*Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate.*" *Advanced Drug Delivery Reviews* **53**(1): 5-21.