Ana Lúcia Morgado Ferreira

Isolamento e caracterização de bactérias acumuladoras de PHAs em HSSL

Isolation and characterization of PHAs-accumulating bacteria from HSSL
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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. Luisa 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

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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.
Polihidroxialcanoatos (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 sulfeto 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 selecionado 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 ($\mu_{\text{max}}$) superior com HSSL como fonte de carbono, $0.212 \pm 0.0219$ e $0.251 \pm 0.0526$ h$^{-1}$ 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, *Alphaproteobactéria*, recorreu-se à construção de uma biblioteca de clones 16S rDNA. Foram identificadas as espécies *Novosphingobium* spp., *Sphingobium* spp e *Pleomorphomonas* spp.
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 ($\mu_{\text{max}}$) when HSSL was used as carbon source, $0.212 \pm 0.0219$ h$^{-1}$ and $0.251 \pm 0.0526$ 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.
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<th>Definition</th>
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<tbody>
<tr>
<td>3HA</td>
<td>3-hydroxyalkanoate</td>
</tr>
<tr>
<td>3HB</td>
<td>3-hydroxybutyrate</td>
</tr>
<tr>
<td>3HHx</td>
<td>3-hydroxyhexanoate</td>
</tr>
<tr>
<td>3HV</td>
<td>3-hydroxyvalerate</td>
</tr>
<tr>
<td>ADF</td>
<td>Aerobic Dynamic Feeding</td>
</tr>
<tr>
<td>C/N</td>
<td>Carbon to Nitrogen Ratio</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
</tr>
<tr>
<td>HSSL</td>
<td>Hardwood Sulphite Spent Liquor</td>
</tr>
<tr>
<td>lcl</td>
<td>Long Chain-Length</td>
</tr>
<tr>
<td>LS</td>
<td>Lignosulphonates</td>
</tr>
<tr>
<td>mcl</td>
<td>Medium Chain-Length</td>
</tr>
<tr>
<td>MMC</td>
<td>Mixed Microbial Culture</td>
</tr>
<tr>
<td>NREL</td>
<td>National Renewable Energy Laboratory</td>
</tr>
<tr>
<td>P(3HB)</td>
<td>Poly(3-hydroxybutyrate)</td>
</tr>
<tr>
<td>P(3HB-co-3HV)</td>
<td>Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)</td>
</tr>
<tr>
<td>P(3HHD)</td>
<td>Poly(3-hydroxyhedecanoato)</td>
</tr>
<tr>
<td>P(3HOD)</td>
<td>Poly(3-hydroxyhexadecanoato)</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>Poly(4-hydroxybutyrate)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PHAs</td>
<td>Polyhydroxyalkanoates</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SBR</td>
<td>Sequencing Batch Reactor</td>
</tr>
<tr>
<td>scl</td>
<td>Short Chain-Length</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
<tr>
<td>VSS</td>
<td>Volatile Suspended Solids</td>
</tr>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$)</td>
<td>Maximum Specific Growth Rate</td>
</tr>
<tr>
<td>$t_d$ (h)</td>
<td>Duplication time</td>
</tr>
<tr>
<td>$q_s$</td>
<td>Substrate Specific Uptake Rate</td>
</tr>
<tr>
<td>$Y_{(X/S)}$</td>
<td>Yield of Biomass on Substrate</td>
</tr>
</tbody>
</table>
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).
This project aims 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 disposable 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).](image)

### 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).

\[
\begin{align*}
\text{(PHA)} & = \text{OCH(CH}_2_n\text{C)}_x \\
R & = \text{alkyl groups (C1 to C13)}
\end{align*}
\]

\[
x = 100 - 30000
\]

<table>
<thead>
<tr>
<th>n</th>
<th>R</th>
<th>PHA</th>
<th>Monomer Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>Poly(3-hydroxypropionate) (P3HP)</td>
<td>Poly(3-hydroxypropionate)</td>
</tr>
<tr>
<td></td>
<td>methyl</td>
<td>Poly(3-hydroxybutyrate) (P3HB)</td>
<td>Poly(3-hydroxyvalerate)</td>
</tr>
<tr>
<td></td>
<td>ethyl</td>
<td>Poly(3-hydroxyhexanoate) (P3HHx)</td>
<td>Poly(3-hydroxyoctanoate)</td>
</tr>
<tr>
<td></td>
<td>propyl</td>
<td>Poly(3-hydroxyhexanoate) (P3HHx)</td>
<td>Poly(3-hydroxyoctanoate)</td>
</tr>
<tr>
<td></td>
<td>pentyl</td>
<td>Poly(3-hydroxyhexanoate) (P3HHx)</td>
<td>Poly(3-hydroxyoctanoate)</td>
</tr>
<tr>
<td></td>
<td>nonyl</td>
<td>Poly(3-hydroxydecaneoate) (P3HHx)</td>
<td>Poly(3-hydroxyoctanoate)</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\text{PHAs} & \text{ are classified on the basis of number of carbon atoms of monomers. There are three classes: short chain length PHAs (scl-PHAs), in which the monomers have between 3 and 5 carbon atoms, medium chain length PHAs (mcl-PHAs), consisting of monomers with 6 to 14 carbon atoms, and long chain length PHAs (lcl-PHAs), monomers with over 14 carbon atoms (Singh et al. 2008).}
\end{align*}
\]

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).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Melting temperature (ºC)</th>
<th>Glass transition temperature (ºC)</th>
<th>Young’s modulus (GPA)</th>
<th>Elongation to break (%)</th>
<th>Tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB)</td>
<td>180</td>
<td>4</td>
<td>3.5</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>53</td>
<td>-48</td>
<td>149</td>
<td>1000</td>
<td>104</td>
</tr>
<tr>
<td>P(3HB-co-20% 3HV)</td>
<td>145</td>
<td>-1</td>
<td>1.2</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>P(3HB-co-16% 4HB)</td>
<td>150</td>
<td>-7</td>
<td>-</td>
<td>444</td>
<td>26</td>
</tr>
<tr>
<td>P(3HB-co-10% 3HHx)</td>
<td>127</td>
<td>-1</td>
<td>-</td>
<td>400</td>
<td>21</td>
</tr>
<tr>
<td>P(3HB-co-6% 3HD)</td>
<td>130</td>
<td>-8</td>
<td>-</td>
<td>680</td>
<td>17</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>176</td>
<td>-10</td>
<td>1.7</td>
<td>400</td>
<td>34.5</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>240</td>
<td>100</td>
<td>3.1</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

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

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 compositied 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>
<thead>
<tr>
<th>Polymer</th>
<th>Trade name</th>
<th>Manufactures</th>
<th>Price (kg⁻¹) (in 2010)</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB) and P(HB-co-HV)</td>
<td>Biomer®</td>
<td>Biomer Inc. (Germany)</td>
<td>3.0 – 5.0 €</td>
<td>-polymer pellets sold commercially for use in classical transformation processes -production of articles (combs, pens and bullets)</td>
</tr>
<tr>
<td>P(3HB)</td>
<td>Mirel™</td>
<td>Metabolix (USA)</td>
<td>1.50 €</td>
<td>-coat paper and paperboards -injection, blow moulding, film production and for electric and electronic packaging -produce shampoo bottles, motor oil bottles and disposable razors</td>
</tr>
<tr>
<td>P(3HB-co-3HHx)</td>
<td>Nodax™</td>
<td>Procter and Gamble (USA)</td>
<td>2.50 €</td>
<td>-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.</td>
</tr>
</tbody>
</table>
2.3 PHAs production process

Bacterial biosynthesis of PHAs is usually in response to stress conditions such as nutrient limitation, for example nitrogen or phosphate with excess carbon source. Bacteria in this group include *Cupriavidus necator* (formerly called *Alcaligenes eutrophus*, *Waustersia eutropha* or *Ralstonia eutropha*). However, some bacteria, such as *Alcaligenes latus*, are able to accumulate PHAs under non-limiting conditions (Akaraonye et al. 2010; Keshavarz et al. 2010).

**Figure 3**: Schematic representation of PHAs biosynthesis from sugar catabolism, fatty acid β-oxidation and intermediary pathways (Gumel et al. 2012).
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 carbons 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; Akaraanye 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; Akaraanye 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).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignosulphonates</td>
<td>78.2 ± 0.6</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>Furfural</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>19.8 ± 0.2</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>24.6 ± 0.5</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>8.5 ± 0.9</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>0.4 ± 0.3</td>
</tr>
</tbody>
</table>
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 selectec 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 describe 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.
Isolation and characterization of PHAs-accumulating bacteria from HSSL

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 *Gamaproteobacteria* using FISH. *Thauera* genus was found to be the dominant genus and *Amaricocus* 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 *Defluvicoccus*, 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).
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 \textit{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 \textit{Firmicutes}, and were identified as \textit{B. subtilis}, \textit{B. badius}, \textit{B. tequilensis}, \textit{Staphylococcus arlettae} and \textit{Enterococcus italicus}. The other two isolates belonged to \textit{Gamaproteobacteria}, \textit{Serratiaureilytica} and \textit{Pseudomonas otitidis} (Venkateswar Reddy et al. 2012a). PHAs production from the isolated \textit{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 \textit{Microlunatus phosphovorus} 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 SO$_2$ 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 (CH$_3$COONa) and Xylose (C$_5$H$_{10}$O$_5$) 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (g.L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monopotassium phosphate (KH(_2)PO(_4))</td>
<td>0.016</td>
</tr>
<tr>
<td>Dipotassium phosphate (K(_2)HPO(_4))</td>
<td>0.064</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate (MgSO(_4)·7H(_2)O)</td>
<td>0.160</td>
</tr>
<tr>
<td>Calcium sulfate dihydrate (CaSO(_4)·2H(_2)O)</td>
<td>0.080</td>
</tr>
<tr>
<td>Iron(III) chloride (FeCl(_3))</td>
<td>0.020</td>
</tr>
<tr>
<td>Sodium Molybdate dihydrate (Na(_2)MoO(_4)·2H(_2)O)</td>
<td>0.008</td>
</tr>
<tr>
<td>Ammonium chloride (NH(_4)Cl)</td>
<td>0.160</td>
</tr>
</tbody>
</table>

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 20ºC. 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\textsubscript{420nm}) into concentration using a calibration curve of Abs\textsubscript{420nm} versus biomass concentration, in VSS (g.L\textsuperscript{-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 then 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 ng.µL⁻¹) were added to the wells and the slide placed in the hybridization chamber at 46ºC 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 48ºC 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.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’ – 3’)</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCTGCCTCCCCGTAGGAGT</td>
<td><em>Bacteria</em></td>
<td>(Amann et al. 1995)</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>GCAGCCACCCGTAGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUB338 III</td>
<td>GCTGCCACCCGTAGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alf968</td>
<td>GGTAAGGTTCTGCAGTT</td>
<td><em>Alphaproteobacteria</em> (except <em>Rickettsiales</em>)</td>
<td>(Neef 1997)</td>
</tr>
<tr>
<td>Bet42a</td>
<td>GCCTCCCCACTTCGTTT</td>
<td><em>Betaproteobacteria</em></td>
<td>(Manz et al. 1992)</td>
</tr>
<tr>
<td>Gam42a</td>
<td>GCCTCCCCACATCGTTT</td>
<td><em>Gammaproteobacteria</em></td>
<td>(Manz et al. 1992)</td>
</tr>
<tr>
<td>Hgc69a</td>
<td>TATAGTTACCACCGCGCT</td>
<td><em>Actinobacteria</em> (high GC Gram+ bacteria)</td>
<td>(Roller et al. 1994)</td>
</tr>
<tr>
<td>Sph120</td>
<td>GGGCAGATCCACCACCGCT</td>
<td><em>Sphingomonadales</em></td>
<td>(Eilers et al. 2000)</td>
</tr>
</tbody>
</table>
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 µl 2x buffer A (200 mM Tris, pH 8.0, 50 mM EDTA, 200 mM NaCl, 2 mM sodium citrate, 10 mM CaCl2) and 30 µl 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 µl proteinase K (20 mg.mL⁻¹) and 10 µl 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 µl phenol/chloroform/isoamyl alcohol (24:24:1), 200 µl 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 ml 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 µl 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 µL 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 µL) 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 ml 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:
- 94°C 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:
- 95°C 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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>27f</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
</tr>
<tr>
<td>1492r</td>
<td>TACGGYTCCTGTACGGACTT</td>
</tr>
<tr>
<td>T7f</td>
<td>TAATACGACTCACTATAGGG</td>
</tr>
<tr>
<td>U19r</td>
<td>GTTTTCCCGGTCACGACGT</td>
</tr>
<tr>
<td>M13r</td>
<td>TCACACGAAACAGCTATGAC</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
</tr>
<tr>
<td>530f</td>
<td>GTGCCAGCMGCCGCGCG</td>
</tr>
<tr>
<td>926f</td>
<td>AAACGAAKGAATTGACGG</td>
</tr>
<tr>
<td>907r</td>
<td>CCGTCAATTCMTTTRAGTTT</td>
</tr>
<tr>
<td>519r</td>
<td>GWATTACCGCGCGGCTG</td>
</tr>
</tbody>
</table>

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.
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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 coccobacilus 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.

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Nile Blue Stain (Phase Contrast / Epifluorescence)</th>
<th>Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AF1</strong> Bacilli</td>
<td></td>
<td>Complete Sequencing: 100% identity with <em>Rhodococcus</em> spp.</td>
</tr>
<tr>
<td>Gram variable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF2 Bacilli</td>
<td></td>
<td>Partial Sequencing: 100% identity with <em>Rhodococcus</em> spp.</td>
</tr>
<tr>
<td>Gram variable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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**AF3 Coccobacilli**
- Gram(-)
- Complete Sequencing: 99% identity with *Pseudomonas* spp.

**AF4 Coccobacilli**
- Gram(-)
- Partial Sequencing: 100% identity with *Pseudomonas* spp.

**AF5 Coccobacilli**
- Gram(-)
- Partial Sequencing: 100% identity with *Pseudomonas* 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
Isolation and characterization of PHAs-accumulating bacteria from HSSL

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,
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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 triplicated. 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.

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

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
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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±0.0966 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±0.0538 gVSS.L\(^{-1}\), and seventeen times higher than using acetic acid, 0.0308±0.00247 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 g COD.L\(^{-1}\) than xylose 0.124 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 (\(\mu_{\text{max}}\)) of 0.212±0.0219 h\(^{-1}\) corresponding to duplication time of 3.29±0.344 h. This \(\mu_{\text{max}}\) was the highest showed by this strain. With acetic acid as sole carbon source AF1 presented a \(\mu_{\text{max}}\) of 0.153±0.0252 h\(^{-1}\) corresponding to duplication time of 4.61±0.800h and in xylose a \(\mu_{\text{max}}\) of 0.188±0.0238 h\(^{-1}\) corresponding to duplication time of 3.73±0.504h. 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).
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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 (C).
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 ± 0.0297 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±0.0121 gVSS.L⁻¹ and 0.0642±0.0124 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 $\mu_{\text{max}}$ in the essays with HSSL, 0.212±0.0219h⁻¹ and 0.251±0.0526h⁻¹, presented by strains AF1 and AF3 respectively, as well as the biomass yield from substrate (Y($X/S$)), which were 0.454 gX.gS⁻¹ and 0.747 gX.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 0.572gCOD.gVSS⁻¹.h⁻¹ and 0.430gCOD.gVSS⁻¹.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 $\mu_{\text{max}}$ of 0.130 h⁻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 $\mu_{\text{max}}$ of 0.143 h⁻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 $\mu_{\text{max}}$ 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 ($\mu_{\text{max}}$) range of 0.15–0.62 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 * Scheffersonmyces stipiti stipitis* (Pereira et al. 2012).
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**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 9 hours 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 determined in order to confirm that the inclusions correspond to PHAs and evaluate which polymer was accumulated, once both strains were reported in previous studies 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 5-20 g.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, $\mu_{\text{max}}$ 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-\textit{Rhodococcus} spp., taken at different times of growth in liquid medium with three different carbon sources (HSSL, acetic acid and xylose).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nile Blue Staining</th>
<th>t=0h</th>
<th>t=6h</th>
<th>t=9h</th>
<th>t=25h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSSL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**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).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>t=0h</th>
<th>t=2h</th>
<th>t=9h</th>
<th>t=25h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HSSL</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Acetic Acid</strong></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Xylose</strong></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Taxonomic affiliation</th>
<th>Number of clones</th>
<th>Highest similarity</th>
<th>Identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Achromobacter</em> spp.</td>
<td>Betaproteobacteria 5</td>
<td><em>Achromobacter denitrificans</em> DSM 30026</td>
<td>99%</td>
<td>NR_042021.1</td>
</tr>
<tr>
<td><em>Clostridium</em> spp.</td>
<td>Firmicutes 2</td>
<td><em>Clostridium estertheticum</em> DSM 14864</td>
<td>98%</td>
<td>NR_042153.1</td>
</tr>
<tr>
<td><em>Comamonas</em> spp.</td>
<td>Betaproteobacteria 3</td>
<td><em>Comamonas testosterone CNB-2</em></td>
<td>99%</td>
<td>NR_102841.1</td>
</tr>
<tr>
<td><em>Methylobacillus</em> spp.</td>
<td>Betaproteobacteria 6</td>
<td><em>Methylobacillus flagellatus</em> K</td>
<td>99%</td>
<td>NR_074178.1</td>
</tr>
<tr>
<td><em>Novosphingobium</em> spp.</td>
<td>Alphaproteobacteria 2</td>
<td><em>Novosphingobium naphthalenivorans</em> TUT562</td>
<td>96%</td>
<td>NR_041046.1</td>
</tr>
<tr>
<td><em>Pedobacter</em> spp.</td>
<td>Bacteroidetes 1</td>
<td><em>Pedobacter terrae</em> DS-57</td>
<td>95%</td>
<td>NR_044005.1</td>
</tr>
<tr>
<td><em>Pleomorphomonas</em> spp.</td>
<td>Alphaproteobacteria 2</td>
<td><em>Pleomorphomonas koreensis</em> Y9</td>
<td>99%</td>
<td>NR_043997.1</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>Gammaproteobacteria 2</td>
<td><em>Pseudomonas moraviensis</em> CCM 7280</td>
<td>99%</td>
<td>NR_043314.1</td>
</tr>
<tr>
<td><em>Sphingobium</em> spp.</td>
<td>Alphaproteobacteria 3</td>
<td><em>Sphingobium</em> sp. SYK-6</td>
<td>95%</td>
<td>NR_074396.1</td>
</tr>
</tbody>
</table>

*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 $72.7 \pm 4.0 \%$, followed by *Betaproteobacteria*, $11.1 \pm 0.37 \%$ and *Gammaproteobacteria*, $10.3 \pm 0.3 \%$. 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).
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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, nonylphenoletoxylate 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 devided into four genera: Sphingomonas sensu stricto, Sphingobium, Novosphingobium and Sphingopyxis (Takeuchi et al. 2001). Novosphingobium nitrogenifigens Y88T 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<sup>T</sup> 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<sup>T</sup> 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).
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 theirs PHAs production ability (if reported).

<table>
<thead>
<tr>
<th>Studies of Identification / Isolation from Activated Sludge</th>
<th>PHAs-accumulating capability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Achromobacter spp.</strong></td>
<td></td>
</tr>
<tr>
<td><em>Achromobacter xylosoxidans</em> CS5</td>
<td>Biodegradation of endosulfan</td>
</tr>
<tr>
<td></td>
<td><em>(Li et al. 2009)</em></td>
</tr>
<tr>
<td><em>Achromobacter</em></td>
<td>Isolation of nonylphenol ethoxylate-degrading bacteria (Gu et al. 2010)</td>
</tr>
<tr>
<td><em>Achromobacter</em></td>
<td>Biogradation of di-n-butyl phthalate (He et al. 2013)</td>
</tr>
<tr>
<td>sp.</td>
<td></td>
</tr>
<tr>
<td><strong>Clostridium spp.</strong></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em> CL3</td>
<td>Hydrogen production from glycerol (Mangayil et al. 2012)</td>
</tr>
<tr>
<td><em>Clostridium subterminale</em> DSM 758</td>
<td><em>Clostridium botulinum</em> ATCC 9564 <em>(Emeruwa et al. 1973)</em></td>
</tr>
<tr>
<td><em>Clostridium</em> sp.</td>
<td>Study microbial community in printing and dyeing wastewater treatment system (Yang et al. 2012)</td>
</tr>
<tr>
<td><strong>Comamonas spp.</strong></td>
<td></td>
</tr>
<tr>
<td><em>Comamonas badia</em></td>
<td>Activated sludge system treating coking effluent (Felföldi et al. 2010)</td>
</tr>
<tr>
<td></td>
<td><em>Comamonas</em> sp. EB172 <em>(Zakaria et al. 2010)</em></td>
</tr>
<tr>
<td><em>Comamonas sp.</em></td>
<td>Assessment of denitrifying bacterial composition in activated sludge (Srinandan et al. 2011)</td>
</tr>
<tr>
<td></td>
<td><em>Comamonas testosteroni</em> <em>(Thakor et al. 2005)</em></td>
</tr>
<tr>
<td><em>Comamonas acidovorans</em></td>
<td>Biodegradation of phenol (Safont et al. 2012)</td>
</tr>
</tbody>
</table>
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 theirs PHAs production ability (if reported).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Main Objective</th>
<th>Ref.</th>
<th>PHAs-accumulating capability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylobacillus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spp.</td>
<td>Identification of Acetate- or Methanol-Assimilating Bacteria</td>
<td>(Osaka et al. 2006)</td>
<td></td>
</tr>
<tr>
<td><strong>Methylobacillus</strong></td>
<td>antibiotics removal</td>
<td>(Xia et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Novosphingobium</strong></td>
<td>Isolation of an β-estradiol-degrading bacterium</td>
<td>(Fujii et al. 2003)</td>
<td>Novosphingobium nitroenifigens sp. Y88^T</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td>(Addison et al. 2007)</td>
</tr>
<tr>
<td>ARI-1^T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Novosphingobium</strong></td>
<td>Estrogen removal</td>
<td>(Hashimoto et al. 2012)</td>
<td>Novosphingobium sp. THA_AIK7</td>
</tr>
<tr>
<td>sp. JEM-1</td>
<td></td>
<td></td>
<td>(Teeka et al. 2012)</td>
</tr>
<tr>
<td><strong>Pedobacter</strong></td>
<td>Biodegradation of 2-chloro-nitrobenzene</td>
<td>(Xu et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pleomorphomonas</strong></td>
<td>Treatment of linear alkylbenzene sulfonate domestic wastewater treatment</td>
<td>(Duarte et al. 2010)</td>
<td>Pleomorphomonas oryzae</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td>(Xie et al. 2005)</td>
</tr>
<tr>
<td>sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sphingobium</strong></td>
<td>Bioegradation of pyrethroid</td>
<td>(Guo et al. 2009)</td>
<td>Sphingobium scionense sp. WP01^T</td>
</tr>
<tr>
<td>spp.</td>
<td></td>
<td></td>
<td>(Liang et al. 2010)</td>
</tr>
<tr>
<td>JZ-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spp.</td>
<td>Isolation of nonylphenol ethoxylate-degrading bacteria</td>
<td>(Gu et al. 2010)</td>
<td></td>
</tr>
</tbody>
</table>
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.](image)
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 responsible 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 $\mu_{\text{max}}$, of 0.212±0.0219 h⁻¹ and 0.251±0.0526 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 0.572gCOD.gVSS⁻¹.h⁻¹ and 0.430gCOD.gVSS⁻¹.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


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