

# Polyhydroxyalkanoates-accumulating bacteria isolated from activated sludge acclimatized to hardwood sulphite spent liquor

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**Abstract** This work aimed to isolate and characterize organisms able to store polyhydroxyalkanoates (PHAs), from a mixed microbial culture (MMC) selected under feast and famine conditions, using hardwood sulfite spent liquor (HSSL), a by-product of the pulp and paper industry, as the carbon source. The MMC was able to accumulate PHA from the different HSSL carbon components (acetic acid, xylose and lignosulfonates) with a PHA storage content during the reactor operation of  $54.2 \pm 10.6$  % PHB. In order to clarify the MMC composition, isolation was performed from the MMC, using HSSL or its main components. Three isolates were identified: *Rhodococcus* spp., *Pseudomonas* spp. and *Klebsiella* spp. All isolates were able to grow and accumulate PHAs in harsh conditions of HSSL using xylose, the second most abundant sugar in nature. *Rhodococcus* and *Pseudomonas* isolates were able to accumulate a homopolymer PHB, whereas *Klebsiella* accumulated the copolymer P(HB-co-HV), with a composition of 96 % HB and 4 % HV. 16S rDNA clonal analysis was performed to identify the unculturable fraction of the MMC, and members of *Novosphingobium* spp., *Sphingobium* spp. and *Pleomorphomonas* spp., were found to be related to PHA production. The capability of the isolated bacteria to simultaneously consume xylose and accumulate PHAs offers a possibility for further studies and routes for the valorization of xylose-rich by-products.

**Keywords** Polyhydroxyalkanoates · Mixed microbial culture · Hardwood sulfite spent liquor · Microbial isolation

## Introduction

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 of conventional plastics (Gumel et al. 2013). In the last few years, the interest in polyhydroxyalkanoates production by mixed microbial cultures (MMC), such as activated sludge from wastewater treatment plants, has been growing, since their use does not require sterile conditions, thus saving energy and equipment costs (Serafim et al. 2008; Villano et al. 2010). In this way, overall production costs can be further decreased. The selection of substrate is an important factor to optimize PHAs production since it affects the final cell content, composition and properties of the polymer. Moreover, raw materials can account for 40 % of the total operating costs of PHAs production, and more than 70 % of these costs are related to the carbon source (Reis et al. 2003). Consequently, the use of cheap carbon substrates is attracting considerable interest (Serafim et al. 2008; Akaraonye et al. 2010). Recently, Queirós et al. (2014) successfully selected a MMC, which under feast and famine conditions was able to store PHAs from hardwood sulfite spent liquor (HSSL), a by-product of the pulp and paper industry.

Despite the growing importance of PHAs production by MMCs, numerous researchers have been attempting to isolate PHAs-producing microorganisms from different sources aiming to discover and identify robust and novel species with high storage capacity (Sangkharak and Prasertsan 2012). The isolation of PHAs-accumulating bacteria from activated

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sludge has been the object of several studies (Liu et al. 2000; Wong et al. 2002; Kourmentza et al. 2009; Reddy and Mohan 2012). The search for strains able to resist and metabolize inhibitory compounds present in sugarcane bagasse hydrolysates was performed by Lopes et al. (2014). *Burkholderia* sp. F24 reached values of biomass of  $25 \text{ g L}^{-1}$ , with a content of 49 % polyhydroxybutyrate (PHB) in 44 h, corresponding to a volumetric productivity of  $0.28 \text{ g L}^{-1} \text{ h}^{-1}$  (Lopes et al. 2014).

Therefore, the present paper aims to study the microbial population selected by Queirós et al. (2014) in order to improve the use of HSSL as a substrate in the biorefinery concept. The isolation and characterization of organisms from the MMC was performed to obtain PHAs-accumulating bacteria able to grow under harsh conditions brought by complex substrates, such as HSSL, and to use xylose as carbon source. Additionally, a 16S rRNA gene clonal analysis was carried out in order to identify the uncultivable PHAs-accumulating bacteria present in the MMC.

## Materials and methods

### Culture

Microorganisms isolated in this work were obtained from a PHAs-storing MMC selected in a sequencing batch reactor (SBR) under aerobic dynamic feeding (ADF) with HSSL as substrate. The operation of SBR was described by Queirós et al. (2014); however, it was not fully optimized, showing evidence of a more stable behavior by the end of the operation. Briefly, the SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal wastewater treatment plant, Aveiro Norte (SIMRia). The SBR, with a working volume of 1.5 L, was operated under ADF conditions, during which alternating feast and famine phases were imposed. The SBR worked in cycles of 12 h that comprised 10.5 h of aerobiosis, with fresh medium supplied during the first 15 min, 1 h of settling (with agitation and aeration switched off) and 0.5 h of withdrawing. The hydraulic retention time (HRT) and sludge retention time (SRT) were 1 and 5 days, respectively.

### Culture medium

HSSL was supplied by Caima–Indústria de Celulose (Constância, Portugal) and was obtained from a magnesium-based acidic sulfite pulping of *Eucalyptus globulus* and pretreated as previously described (Xavier et al. 2010; Queirós et al. 2014). The HSSL composition was fully characterized by Marques et al. (2009).

Two different culture media were used to isolate the different microorganisms responsible for the uptake of different substrates. The first culture medium had the same composition of the one fed to the selected MMC in the SBR: pre-

treated HSSL and mineral solution (Queirós et al. 2014). The second one consisted of synthetic mineral, salts and vitamins medium (MSV), prepared with the major carbon sources [sodium acetate ( $\text{CH}_3\text{COONa}$ ) –  $0.172 \text{ g L}^{-1}$  and xylose ( $\text{C}_5\text{H}_{10}\text{O}_5$ ) –  $0.360 \text{ g L}^{-1}$ ] present in HSSL (Table 1). The MSV medium was also composed by (per liter of MilliQ water): 85 mg  $\text{KH}_2\text{PO}_4$ , 110 mg  $\text{K}_2\text{HPO}_4$ , 100 mg  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 80 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2 mg  $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ , 3 mg Na EDTA and 500 mg  $(\text{NH}_4)_2\text{SO}_4$ . An amount of  $1 \text{ mL L}^{-1}$  of vitamin solution, composed by (per liter of MilliQ water): 20 mg Biotin, 20 mg Folic acid, 100 mg Pyridoxine, 50 mg Thiamine, 50 mg Riboflavin, 50 mg Nicotinic acid, 50 mg Pantothenic acid, 2 mg Vitamin  $\text{B}_{12}$ , and 50 mg 4-Aminobenzoic acid, was added to the growth medium. Agar was used to a concentration of  $15 \text{ g L}^{-1}$ .

### Bacterial isolation

In order to isolate PHAs-accumulating bacteria from the selected MMC, solid culture medium was prepared, for each media described above. Despite SBR being operated without temperature control, the temperature remained around  $20 \text{ }^\circ\text{C}$ . Therefore, a volume of  $50 \text{ } \mu\text{L}$  of MMC were spread onto agar growth medium and incubated at  $20 \text{ }^\circ\text{C}$  for 48 h. Pure cultures of PHAs-storing bacteria were isolated after repeated streaking of isolated colonies. The PHAs-accumulating capacity of the isolates was evaluated through their inoculation on 50-mL Erlenmeyer flasks with culture medium of  $0.5 \text{ g L}^{-1}$  of acetic acid or xylose, at  $20 \text{ }^\circ\text{C}$  for 48 h followed by Nile blue staining of samples.

### Kinetic tests

A 400-mL Erlenmeyer flask was inoculated with 50 mL of actively growing cells on culture medium plus the carbon source with the same concentration as in SBR feeding:  $0.172 \text{ g L}^{-1}$  of sodium acetate or  $0.360 \text{ g L}^{-1}$  of xylose.

**Table 1** Composition of eucalypt sulfite spent liquor (Xavier et al. 2010)

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

Kinetic tests were performed at 20 °C, 90 rpm for 24 h, in triplicate. Samples were taken during tests to evaluate the culture growth and substrate consumption through chemical oxygen demand (COD) analysis. Nile blue staining procedure was performed in order to evaluate PHAs accumulation.

### Microscopic visualizations

Gram-staining was performed according to the procedure described by Jenkins et al. (1986). Nile blue staining was applied to fresh samples collected as described by Rees et al. (1992), with the goal of monitoring the PHAs accumulating capacity of the isolates during the incubations.

Fluorescence in situ hybridization (FISH) was performed on paraformaldehyde-fixed biomass samples (Amann et al. 1995). Several oligonucleotide probes were applied. All the hybridizations with group-specific probes were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. All the probes were synthesized with 50-FITC and -Cy3 labels and purchased from MWG Biotech (Germany). Samples were visualized using an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

### Analytical methods

Overall substrate consumption was determined based on COD evolution, using Spectroquant<sup>®</sup> photometric kit (Merck), according to the manufacturer's instructions.

The amount of biomass was accounted as cell dry weight. A sample of 50 mL was filtered using previously dried and weighed membranes (cellulose acetate filter, 0.2 µm pore size; Sartorius). Then, the membranes were placed in an oven at 105 °C for 72 h. After cooling and weighing, the biomass concentration was determined in g L<sup>-1</sup> of suspended solids (SS).

PHA concentration was determined by gas chromatography following the method described by Lemos et al. (2006).

### Genomic DNA extraction and PCR amplification of 16S rRNA gene

DNA was extracted from 2 mL of the MMC sample following the protocol reported in Rossetti et al. (2003). The concentration and purity of the genomic DNA were determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

16S rRNA genes of the isolates and genomic DNA were amplified using primers 27 F and 1492R for the Bacteria domain using the TaKaRa Ex Taq<sup>™</sup> kit (Japan) as previously described (Rossetti et al. 2003). T7f and U19r (or M13r),

which are specific plasmid primers, were used for the screening of clones from the 16S rDNA clone library (Table 2). PCR products were purified using the QIAquick<sup>®</sup> PCR purification kit (Qiagen, Milan, Italy). 16S rRNA gene sequences of the clone inserts were obtained using the following primers: 530f, 926f, 907r and 519r (Table 2).

### Cloning of 16S rRNA gene

Cloning of PCR products was carried out using pGEM-T Easy Vector System (Promega, USA) into *Escherichia coli* JM109 competent cells (Promega) according to the manufacturer's instructions. Positive inserts were amplified from recombinant plasmids obtained from white colonies by PCR using the sequencing primers T7f and M13r following the PCR protocol previously described.

### Nucleotide sequences accession numbers

The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers KM873626, KM873627 and KM873628, for the isolates AF1, AF2 and DS1, respectively. For the clones FA1, FA2, FA3, FA4, FA5 and FA6, the accession numbers were KM873629, KM873630, KM873631, KM873632, KM873633 and KM873634, respectively.

## Results and discussion

### SBR operation

The MMC used in this study was selected by operating a SBR under ADF conditions with HSSL as substrate, with an organic loading rate (OLR) of 4.2 g COD L<sup>-1</sup> day<sup>-1</sup>, during 67 days without any interruptions. The SBR was inoculated with activated sludge obtained from the aerobic tank of the municipal wastewater treatment plant, Aveiro Norte (SIMRia). The SBR

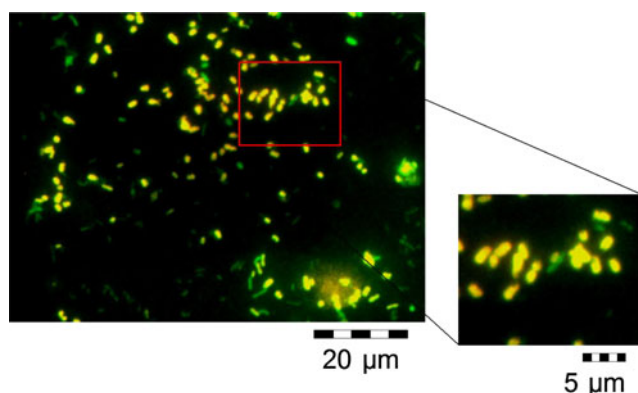
**Table 2** Primers used in PCR and sequencing

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

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

worked in cycles of 12 h, with a hydraulic retention time (HRT) of 1 day and a sludge retention time (SRT) of 5 days (Queirós et al. 2014). Queirós et al. (2014) described the SBR operation in more detail and the evolution of the microbial community. They observed that Alphaproteobacteria was clearly the dominant class (Fig. 1) at the end of the reactor operation, when the SBR reached a pseudo-steady state. This observation was based on the stabilization of polymer accumulation in the last 20 days of operation,  $53.25 \pm 3.25$  % PHA. For the same period, a stable microbial populations was observed with minor fluctuations of Alphaproteobacteria,  $75.33 \pm 2.44$  % of the total bacteria. To a minor extent, cells belonging to Betaproteobacteria and Gammaproteobacteria were also detected by FISH analysis. The MMC showed a quite high PHA storage capacity with an average value of storage content of  $54.2 \pm 10.6$  % PHB (Queirós et al. 2014). Hence, the isolation of microorganisms able to consume xylose, accumulate PHA and survive in such harsh conditions was important to understand the community dynamics and establish a starting point to a possible process of PHA production. As xylose is the second most abundant sugar in nature, it is important to indicate microorganisms able to convert it into other value-added products. Moreover, with the increasing research in using lignocellulosic biomass as substrate for several biological processes, those microorganisms are gaining importance.

During the SBR cycles, the concentrations of main carbon sources of HSSL decreased along with ammonium. The MMC revealed a preference towards acetic acid, being completely consumed after 4 h of reaction at a rate of  $0.110 \text{ g L}^{-1} \text{ h}^{-1}$ . The ammonium uptake rate was faster before the acetic acid exhaustion,  $0.177 \text{ g L}^{-1} \text{ h}^{-1}$  decreasing to  $0.074 \text{ g L}^{-1} \text{ h}^{-1}$ . Xylose was consumed along with acetic acid but at a slower rate, around  $0.030 \text{ g L}^{-1} \text{ h}^{-1}$ , but never depleted, remaining around 70 % at the end of each cycle. Concerning the lignosulfonates, there was not a clear tendency in any of the cycles, but it was consumed at a rate of  $0.098 \text{ g L}^{-1} \text{ h}^{-1}$  (Queirós et al. 2014). Figure 2 shows an



**Fig. 1** FISH micrograph taken from SBR at the end of operation. Cells in yellow hybridized with the Alphaproteobacteria-specific probe (ALF968 probe) and green cells hybridized with EUB338mix probes (Bacteria domain)

example of an SBR cycle, in which the accumulation reached 19.5 %, with an  $Y_{\text{PHA/S}}$  of  $0.44 \text{ g PHA g substrate}^{-1}$  (S). Considering only acetic acid as the main precursor for PHA production, the  $Y_{\text{PHA/Acet}}$  increased to 1.04, suggesting that this substrate was not the only one involved in PHA accumulation. This was confirmed by Queirós et al. (2014) during batch tests with acetic acid and xylose fed separately. In these tests, the MMC was able to accumulate PHA from both carbon sources, reaching storage contents of 63.4 and 31.5 %, respectively (Queirós et al. 2014).

### Characterization of the isolates and PHAs storage

The isolation attempts of PHAs-accumulating bacteria able to survive in harsh conditions were first performed on solid medium containing the same composition of that supplied to the SBR. From two different Petri dishes inoculated with the MMC, five isolated colonies from around 150 grown were selected randomly, continuously streaked onto solid medium and named AF1 to AF5. Additionally, synthetic mineral medium, MSV, containing sodium acetate or xylose, the main carbon components of HSSL, was tested. Colonies only grew with xylose as the sole carbon source. After several replating cycles, a pure culture was obtained, and called DS1.

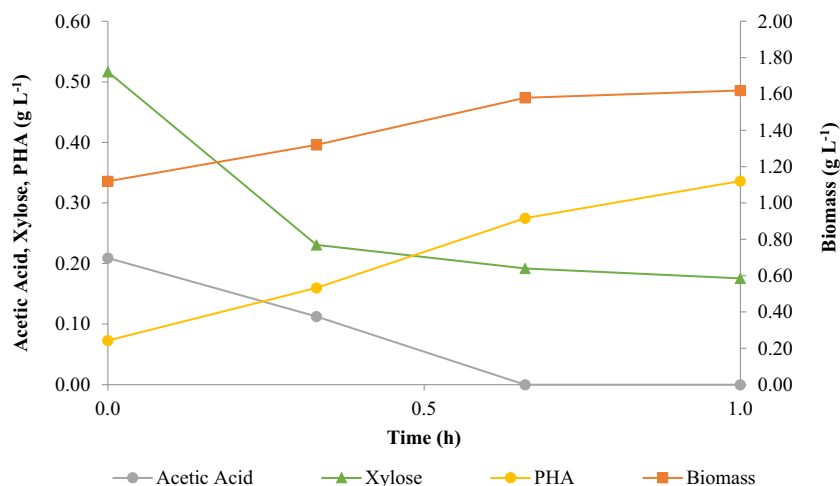
Partial sequencing of 16S rRNA genes of AF1 and AF3 isolates showed 100 % similarity with *Rhodococcus* spp. while isolates AF2, AF4 and AF5 had 100 % similarity with *Pseudomonas* spp. The complete sequencing of each representative of isolates belonging to *Rhodococcus* spp. (AF1) and to *Pseudomonas* spp. (AF2) was performed, and hereafter experiments were performed only using AF1 and AF2 isolates. Near-full-length 16S rRNA gene sequence of AF1 showed that this isolate was closely related to *Rhodococcus qingshengii* strain djl-6, with 100 % of sequence similarity (Xu et al. 2007). The AF2 isolate had 99 % sequence similarity to *Pseudomonas libanensis* strain CIP 105460 (Dabboussi et al. 1999). The complete sequencing of 16S rRNA genes of DS1 isolate allowed for its identification as *Klebsiella* spp., sharing 99 % similarity with an uncultured *Klebsiella* sp. clone JXS1-28 (Peng et al. 2012).

The PHAs accumulating ability of the isolates was evaluated after their inoculation on mineral base medium with sodium acetate at a concentration of  $0.5 \text{ g L}^{-1}$  followed by Nile blue staining of samples. This specific staining for PHAs inclusions confirmed that the isolates were PHAs-accumulating bacteria. The presence of inclusion bodies of PHAs was easily observed by the presence of bright dots in the cells after the Nile blue staining procedure under epifluorescence microscopy (Fig. 3).

Kinetic tests were performed in order to characterize isolates AF1, AF2 and DS1 and evaluate growth on different carbon sources and PHAs-accumulating behavior, namely on HSSL and xylose, the main component of HSSL and a



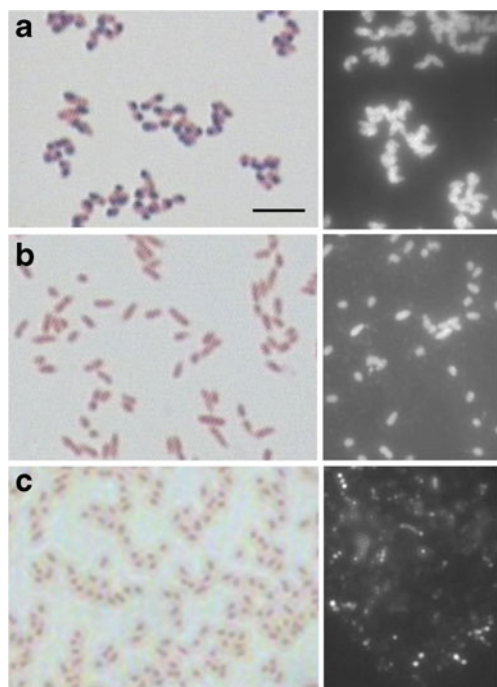
**Fig. 2** SBR cycle example. In each cycle, the acetic acid, xylose, PHA and biomass evolution was followed



carbon source with limited use among microorganisms (Lopes et al. 2009). The results of the kinetic parameters obtained are summarized in Table 3. Growth and substrate consumption in the three different media, for AF1 and AF2, are shown in Figs. 4 and 5, respectively. Diversely from isolates AF1 and AF2, acetate did not sustain the growth of DS1, which only grew on xylose as sole carbon source (Fig. 6). Members of *Klebsiella* were described as able to grow on xylose and to store PHAs and are often found in activated sludge systems (Lopes et al. 2009).

Isolate AF1 showed a preference for medium containing HSSL or xylose, since it began to grow right after inoculation

with an undetectable lag phase in both media. With acetic acid as sole carbon source, the isolate presented a lag phase of around 2 h. A higher biomass concentration, of  $0.510 \pm 0.0966 \text{ g VSS L}^{-1}$ , was obtained in the assay with HSSL (Fig. 4). This value was 6 times higher than the biomass concentration obtained using xylose as sole carbon source,  $0.0867 \pm 0.0538 \text{ g VSS L}^{-1}$ , and 17 times higher than using acetic acid,  $0.0308 \pm 0.00247 \text{ g VSS L}^{-1}$ . One reason for the differences observed in biomass amount may be the lower concentration of substrate in media with acetic acid and xylose, since the amount of carbon sources were determined in order to be approximately the same as in HSSL but other carbon compounds are missing; sugars like mannose, galactose, arabinose and phenolic components such as pyrogallol and galic acids that could be metabolized by the organisms. However, xylose and acetic acid were still present at the end of the kinetic tests, with acetic acid at much lower concentration ( $0.0467 \text{ g COD L}^{-1}$ ) than xylose ( $0.124 \text{ g COD L}^{-1}$ ), with cells already in the death phase, showing that other nutrients besides carbon were missing. The lack of other components present in HSSL needed for microbial growth could be supported by the value of specific growth rate of isolate AF1 when grown in liquid medium with HSSL,  $0.212 \pm 0.0219 \text{ h}^{-1}$ , which was the highest showed by this isolate. With acetic acid as sole carbon source, AF1 presented a maximum growth rate ( $\mu_{\max}$ ) of  $0.153 \pm 0.0252 \text{ h}^{-1}$  and in xylose a  $\mu_{\max}$  of  $0.188 \pm 0.0238 \text{ h}^{-1}$ . Growth and PHAs accumulation by *Rhodococcus* strains using acetate as carbon source has already been reported, but the authors only quantified PHAs accumulation content and monomeric composition, without information about growth (Haywood et al. 1990; Hori et al. 2009). However, the effect of acetate concentration on cell growth of a *Rhodococcus* strain, *R. rhodochrous*, has been previously discussed (Honda et al. 1998). According to Honda et al. (1998), cell growth was significantly inhibited at concentrations of acetate above  $3 \text{ g L}^{-1}$ . Below  $2.5 \text{ g L}^{-1}$ , the growth rate was in a range of 0.15 to  $0.16 \text{ h}^{-1}$ , which



**Fig. 3** Gram and Nile blue staining. Appearance of the isolates **a** AF1, **b** AF2 and **c** DS1 after Gram (left) and Nile blue staining showing intracellular PHA storage (right). Bar 5 µm

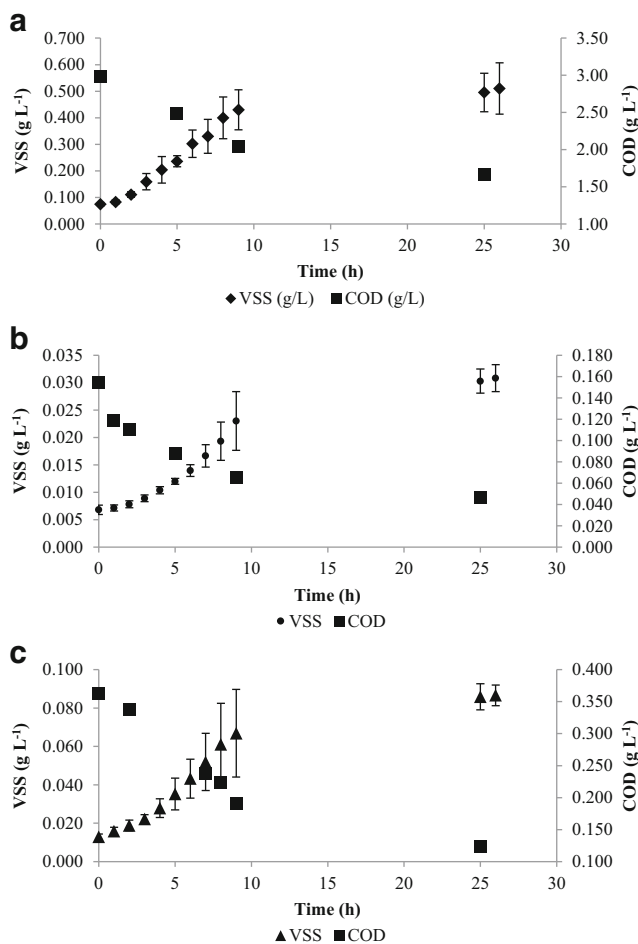
**Table 3** Main parameters obtained from the kinetic batch tests of isolates AF1, AF2 and DS1

Isolate	Carbon source	$\mu_{\max}$ (h <sup>-1</sup> )	$q_s$ (g S g X <sup>-1</sup> h <sup>-1</sup> )	$Y_{(X/S)}$ (g X g S <sup>-1</sup> )
AF1	HSSL	0.212±0.0219	0.466	0.454
	Acetic acid	0.153±0.0252	0.519	0.295
	Xylose	0.188±0.0238	0.572	0.329
AF2	HSSL	0.251±0.0526	0.336	0.747
	Acetic acid	0.194±0.0147	0.430	0.451
	Xylose	0.130±0.00578	0.264	0.492
DS1	Xylose	0.316	1.66	0.191

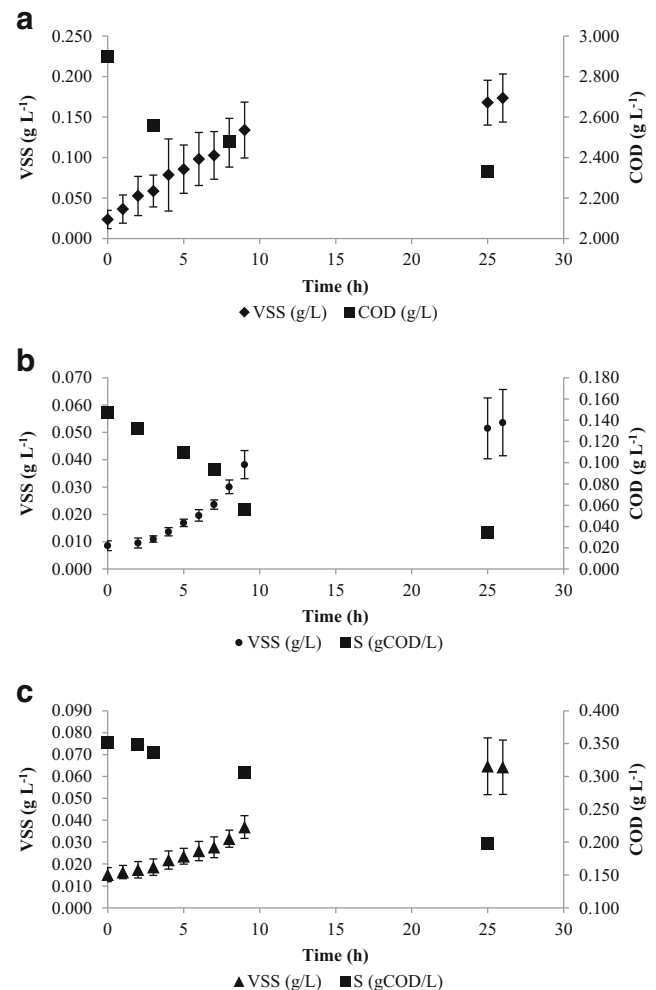
confirmed the results obtained with isolate AF1. The fact that AF1 was able to metabolize xylose was a very interesting finding, since no reports on utilization of this sugar by wild-type strains belonging to genus *Rhodococcus* have thus far been found.

Isolate AF2 identified as *Pseudomonas* sp. showed a similar behavior to AF1, since it also grew in the three media, obtaining higher biomass concentrations when grown in HSSL. However, the biomass concentrations obtained were

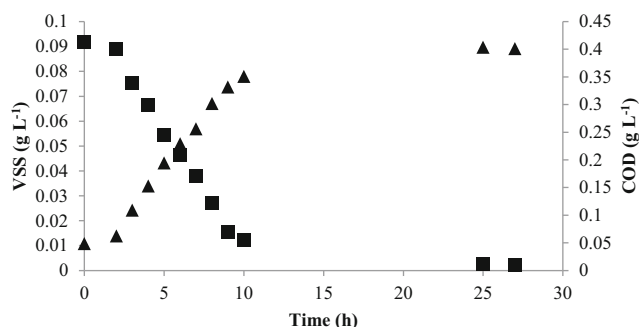
lower than those obtained with AF1. In HSSL, the biomass concentration obtained for AF2,  $0.174\pm 0.0297$  g VSS L<sup>-1</sup>, was three times lower than for AF1 in the same medium. The biomass concentration achieved in HSSL was about three times higher than in the assays using acetic acid or xylose as sole carbon sources, which were  $0.0536\pm 0.0121$  g VSS L<sup>-1</sup> and  $0.0642\pm 0.0124$  g VSS L<sup>-1</sup>, respectively. Table 3 shows



**Fig. 4** Microbial growth evolution and substrate consumption along the kinetic tests performed with isolate AF1 (*Rhodococcus* spp.), in growth media with a distinct carbon source: HSSL (a), acetic acid (b) and xylose (c)



**Fig. 5** Microbial growth evolution and substrate consumption along the kinetic tests performed with isolate AF2 (*Pseudomonas* spp.), in growth media with a distinct carbon source: HSSL (a), acetic acid (b) and xylose (c)



**Fig. 6** Microbial growth evolution and substrate consumption along the kinetic test performed with isolate DS1 (*Klebsiella* spp.) with xylose as carbon source

the preference of both isolates for growing in medium with HSSL: a higher  $\mu_{\max}$  in the essays with HSSL,  $0.212 \pm 0.0219 \text{ h}^{-1}$  and  $0.251 \pm 0.0526 \text{ h}^{-1}$ , and yield biomass on substrate ( $Y_{(X/S)}$ ),  $0.454 \text{ g X g S}^{-1}$  and  $0.747 \text{ g X g S}^{-1}$ , were obtained by AF1 and AF2, respectively. On the other hand, AF1 (*Rhodococcus* spp.) showed a preference in consuming xylose and AF2 (*Pseudomonas* spp.) in acetic acid, as they presented a higher specific substrate consumption rates ( $q_s$ ) of  $0.572 \text{ g COD g VSS}^{-1} \text{ h}^{-1}$  and  $0.430 \text{ g COD g VSS}^{-1} \text{ h}^{-1}$ , respectively, compared to those with the two other substrates. In future studies, quantification of other components of HSSL over time should be performed for a better understanding of the growth and consumption behavior of both isolates.

A qualitative evaluation of the PHAs-accumulating capability of isolates, AF1 and AF2 in liquid medium with three different carbon sources analyzed (HSSL, acetic acid and xylose) was performed (Figs. 4, 5). Samples taken during the kinetics tests were observed under phase contrast and after Nile blue staining. PHAs accumulation by AF1 and AF2 increased during the incubation period reaching a maximum at the late exponential stage of the growth curve. For both isolates, in HSSL and xylose assays, intracellular PHAs inclusions were clearly observed. In acetic acid assay, cells were completely fluorescent, which meant that they were completely full of PHAs. As PHAs accumulation increased and reached the maximum PHAs content, some cells of AF1 and AF2 were completely fluorescent in all tests. Nevertheless, the fluorescence intensity was always higher in assays with acetic acid, meaning that cells of isolates AF1 and AF2 were completely full of PHAs. This finding was somehow expected when acetic acid was the substrate, since volatile fatty acids are the preferred substrates by MMCs for PHAs production (Gumel et al. 2013). In addition, in assays with HSSL, a higher intensity of fluorescence was clearly observed for AF1 than for AF2, which indicates a higher content of PHAs produced by the former. Regarding DS1, this isolate was grown on xylose (Fig. 6). This isolate was also able to produce PHAs and the increase of inclusion bodies during the growth was clearly observed by Nile blue staining.

The identification of PHAs accumulated was performed by GC analysis. The results showed that isolates AF1 and AF2 accumulated a homopolymer PHB, and DS1 accumulated a copolymer poly(hydroxybutyrate-*co*-hydroxyvalerate) [P(HB-*co*-HV)], with 4 % of HV. A large number of different hydroxy-fatty acids may be converted into polymers, but only if suitable carbon sources are provided as precursor substrates. Some substrates lead to monomer formation of equal carbon chain length. This behavior could explain the copolymer accumulation of the isolate DS1 from an unrelated carbon source as xylose. Starting from related substrates, the synthesis pathway is closely connected to the fatty-acid  $\beta$ -oxidation cycle (Babel et al. 2001). In some bacteria, the copolymers P(HB-*co*-HV) are synthesized from sugars by methyl-malonyl-CoA. Succinyl-CoA is decarboxylated via methyl-malonyl-CoA to propionyl-CoA as the precursor of 3-hydroxyvaleryl-CoA (Valentin and Dennis 1996).

The isolation of AF1, AF2 and DS1 can contribute to the development of bioprocesses using xylose as substrate. This sugar is the second most abundant in nature, especially in vegetable biomass, but only a few papers have focused on its valorization and even fewer when it comes to PHA production. As xylose is one of the most abundant sugars in nature, it is important to find microorganisms with the capacity to metabolize it and, consequently, convert it into products of interest.

### 16S rRNA gene clonal analysis

In order to identify the unculturable bacteria responsible for PHAs accumulation of the MMC, a 16S rRNA gene clonal analysis was performed on DNA extracted from the selected MMC. A total of 31 clones were obtained and 26 identified. Clones were closely related to nine different genera: *Achromobacter* spp., *Comamonas* spp., *Clostridium* spp., *Methylobacillus* spp., *Novosphingobium* spp., *Pedobacter* spp., *Pleomorphomonas* spp., *Pseudomonas* spp. and *Sphingobium* spp. (Table 4; Fig. 7). Some of the species highlighted by the clonal analysis have been reported as PHAs-accumulating bacteria and have been previously identified or isolated from activated sludge samples, such as *Clostridium* spp. (Emeruwa and Hawirko 1973), *Comamonas* spp. (Zakaria et al. 2010), *Novosphingobium* spp. (Addison et al. 2007), *Pleomorphomonas* spp. (Xie and Yokota 2005) and *Sphingobium* spp. (Liang and Lloyd-Jones 2010). Dai et al. (2015) also investigated the microbial community evolution when fed with poplar hydrolysates. They also observed enrichments in Alpha- and Betaproteobacteria and identified these classes as mainly responsible for PHB accumulation and suggested further isolation of microorganisms to use wood hydrolysates as substrate for PHA production.

Nearly all the identified bacteria belonging to the acclimatized MMC, both from isolation attempts and the 16S rRNA gene clonal analysis, were consistent with the findings of Queirós

**Table 4** Representation of the taxonomic affiliations of the clones obtained

Taxonomic affiliation	N. of clones	Highest similarity	Identity	Clone	Accession number	
<i>Achromobacter</i> spp.	Betaproteobacteria	5	<i>Achromobacter denitrificans</i> DSM 30026	99 %	FA2	KM873630
<i>Clostridium</i> spp.	Firmicutes	2	<i>Clostridium estertheticum</i> DSM 14864	98 %	FA1	KM873629
<i>Comamonas</i> spp.	Betaproteobacteria	3	<i>Comamonas testosteroni</i> CNB-2	99 % <sup>a</sup>		
<i>Methylobacillus</i> spp.	Betaproteobacteria	6	<i>Methylobacillus flagellatus</i> K	99 % <sup>a</sup>		
<i>Novosphingobium</i> spp.	Alphaproteobacteria	2	<i>Novosphingobium naphthalenivorans</i> TUT562	96 %	FA3, FA4	KM873631, KM873632
<i>Pedobacter</i> spp.	Bacteroidetes	1	<i>Pedobacter terrae</i> DS-57	95 %	FA6	KM873634
<i>Pleomorphomonas</i> spp.	Alphaproteobacteria	2	<i>Pleomorphomonas koreensis</i> Y9	99 % <sup>a</sup>		
<i>Pseudomonas</i> spp.	Gammaproteobacteria	2	<i>Pseudomonas moraviensis</i> CCM 7280	99 % <sup>a</sup>		
<i>Sphingobium</i> spp.	Alphaproteobacteria	3	<i>Sphingobium</i> SYK-6	95 %	FA5	KM873633

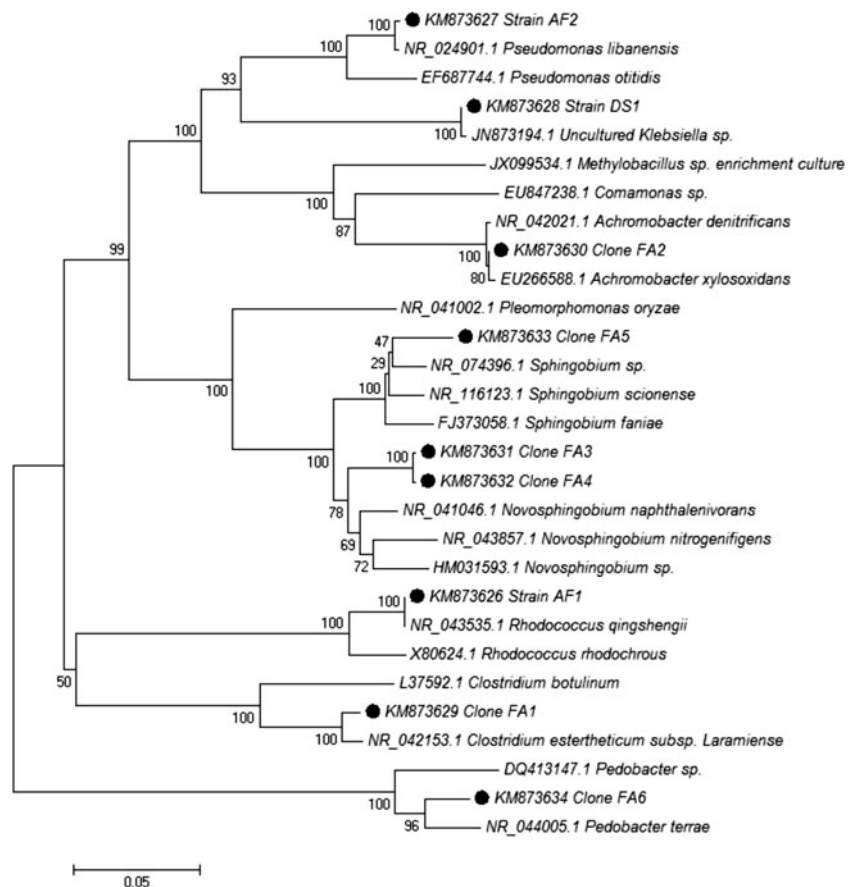
<sup>a</sup>partial sequencing

et al. (2014) after characterization, by FISH, of the acclimatized MMC. Alphaproteobacteria was the dominant group of the MMC, accounting for  $72.7 \pm 4.0$  % of total bacteria, 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 et al. 2014). Nevertheless, a complete characterization of all genera selected in the MMC for an optimum design and modeling of the bioreactor is necessary (Dai et al. 2015). This will allow maximum accumulation and productivity by the MMC.

## Conclusion

Isolates belonging to four of the six groups of the microbial community identified previously were isolated and characterized. It was possible to successfully isolate *Rhodococcus* spp., *Pseudomonas* spp. and *Klebsiella* spp. The isolates were able to both grow and accumulate PHAs despite the harsh conditions brought about by complex substrates, such as HSSL, as well as to use xylose as the carbon source. Moreover, from the clonal analysis, the identification of *Novosphingobium* spp., *Sphingobium*

**Fig. 7** Phylogenetic tree based on full-length nucleotide sequences of 16SrRNA gene of isolate AF1, AF2 and DS1 and clones FA1, FA2, FA3, FA4, FA5 and FA6 (in the tree preceded by a filled dot). The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6





spp. and *Pleomorphomonas* spp. were possible, they having been previously found to be related to PHA production.

This work constitutes a step forward towards the identification of the microorganisms responsible for the accumulation of PHAs in the MMC, and, consequently, to the valorization of HSSL as a complex substrate for biological processes. The isolation of microbial strains able to use xylose, the second most abundant sugar in nature, for PHAs production could be an important advance leading for the valorization of raw materials resulting from the bioprocessing of vegetable biomass.

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**Authors' Contributions** AMF participated in the design of the study, performed the experimental work and wrote the manuscript. DQ was responsible for the isolation of DS1 and its identification and kinetic tests. DQ was also responsible for phylogenetic tree construction and interpretation and the writing of the manuscript. MCG provided technical and scientific guidance. LSS and SR conceived the idea, were part of the design of the study and provided scientific guidance and constructive feedback during the draft of the manuscript. All authors read and approved the final manuscript.

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