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Thauera sp. Sel9, a new bacterial strain for polyhydroxyalkanoates production from volatile fatty acids

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ABSTRACT

Thauera is one of the main genera involved in polyhydroxyalkanoate (PHA) production in microbial mixed cultures (MMCs) from volatile fatty acids (VFAs). However, no *Thauera* strains involved in PHA accumulation have been obtained in pure culture so far. This study is the first report of the isolation and characterization of a *Thauera* sp. strain, namely Sel9, obtained from a sequencing batch reactor (S-SBR) set up for the selection of PHA storing biomass. The 16S rRNA gene evidenced a high sequence similarity with *T. butanivorans* species. Genome sequencing identified all genes involved in PHA synthesis, regulation and degradation. The strain Sel9 was able to grow with an optimum of chemical oxygen demand-to-nitrogen (COD:N) ratio ranging from 4.7 to 18.9. Acetate, propionate, butyrate and valerate were used as sole carbon and energy sources: a lag phase of 72 h was observed in presence of propionate. Final production of PHAs, achieved with a COD:N ratio of 75.5, was 60.12 ± 2.60 %, 49.31 ± 0.7 %, 37.31 ± 0.43 % and 18.06 ± 3.81 % *(w/w)* by using butyrate, acetate, valerate and propionate as substrates, respectively. Also, the 3-hydroxybutyrate/3-hydroxyvalerate ratio reflected the type of carbon sources used: 12.30 ± 0.82 for butyrate, 3.56 ± 0.02 for acetate, 0.93 ± 0.03 for valerate and 0.76 ± 0.02 for propionate. The results allow a better elucidation of the role of *Thauera* in MMCs and strongly suggest a possible exploitation of *Thauera* sp. Sel9 for a cost-effective and environmentally friendly synthesis of PHAs using VFAs as substrate.

Introduction

Polyhydroxyalkanoates (PHAs) are linear polyesters accumulated by prokaryotic microorganisms as intracellular granule reserves of energy and carbon [1]. PHAs have recently gained industrial interest as a promising alternative to non-degradable plastics. This is mainly due to their mechanical, biodegradable and environmentally friendly properties [2]. The most widely studied PHA polymer is poly-(3--hydroxybutyrate) (PHB), a highly crystalline, rigid and friable polymer. However, its mechanical and physical properties can be improved by incorporating 3-hydroxyvalerate (3HV) into the polymer chain [3]. The synthesis of copolymers is a common strategy to increase the mechanical properties of PHAs such as plastics flexibility and lower melting and glass transition temperatures. For instance, poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) copolymers are commonly used for packaging, showing either high tensile strength or high elongation at break [4]. The metabolism of PHAs is well conserved within PHB-accumulating *Betaproteobacteria* [5]. Three genes are involved in PHA synthesis: acetyl-coenzyme A (CoA) acetyltransferase (*phaA*), acetoacetyl-CoA reductase (*phaB*), and PHA polymerase (*phaC*) [6,7]. Moreover, *phaP* (phasin) and *phaR* (transcriptional repressor of *phaP*) were identified as playing an important role in regulation of PHA granule formation. PhaP is a soluble protein in the cytoplasm that become insoluble when it binds to the PHA granules. Phasin is the major structural protein of the PHA granule surface layer. Eventually, *phaZ* is responsible for the depolymerization of PHB stored in the bacteria inclusions [8].

PHA production is induced by high concentration of carbon-

Abbreviations: 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; Ac, acetate; AU, arbitrary units; But, butyrate; COD, chemical oxygen demand; DM, defined medium; MLSSs, mixed liquor suspended solids; MMC, microbial mixed culture; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); PHV, poly(3-hydroxyvalerate); PHBHV, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); Pr, propionate; RFI, relative fluorescence intensity; S-SBR, sequencing batch reactor; SRT, sludge retention time; TEM, transmission electronic microscopy; TSA, tryptone soya agar; TSB, tryptone soya broth; Val, valerate; VFAs, volatile fatty acids.

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Table 1
Protein equivalents for PHA synthesis, regulation and degradation observed in the genome of *Thauera* sp. Sel9. All the related proteins were identified in strain *T. butanivorans* NBRC 103042T.

Category	Protein	Gene annotation	Acc. Number	Number of amino acids	Most related protein - *T. butanivorans* NBRC 103042T -	Accession no.	Identity (%)
Synthesis	PhaA	Acetyl-CoA Acetyltransferase	NX068_14955	428 aa	Acetyl-CoA C-acetyltransferase	WP_068638820	97.66
			NX068_07310	403 aa	Acetyl-CoA C-acetyltransferase	WP_068636275	96.03
	PhaB	Acetoacetyl-CoA Reductase	NX068_11230	246 aa	Acetoacetyl-CoA Reductase	WP_068637009	99.59
		Beta-ketoacyl-ACP reductase	NX068_11235	246 aa	Beta-ketoacyl-ACP reductase	WP_068637010	97.97
	PhaC	Class I poly(R)-hydroxyalkanoic acid synthase	NX068_07285	608 aa	Class I poly(R)-hydroxyalkanoic acid synthase	WP_068636270	98.19
			NX068_11225	581 aa	Class I poly(R)-hydroxyalkanoic acid synthase	WP_068637008	95.52
Surface Proteins	PhaP	Phasin family protein	NX068_12530	188 aa	Phasin family protein	WP_068639241	100
			NX068_10460	167 aa	Phasin family protein	WP_068636571	99.40
			NX068_16290	147 aa	Phasin family protein	WP_068638328	100
Gene regulation	PhaR	Polyhydroxyalkanoate synthesis repressor	NX068_11240	199 aa	Polyhydroxyalkanoate synthesis repressor	WP_068637011	95.48
Degradation	PhaZ	PHB depolymerase family esterase	NX068_05920	341 aa	PHB depolymerase family esterase	WP_068638925	95.04
		Poly(3-hydroxybutyrate) depolymerase	NX068_13455	351 aa	Poly(3-hydroxybutyrate) depolymerase	WP_245650896	97.96

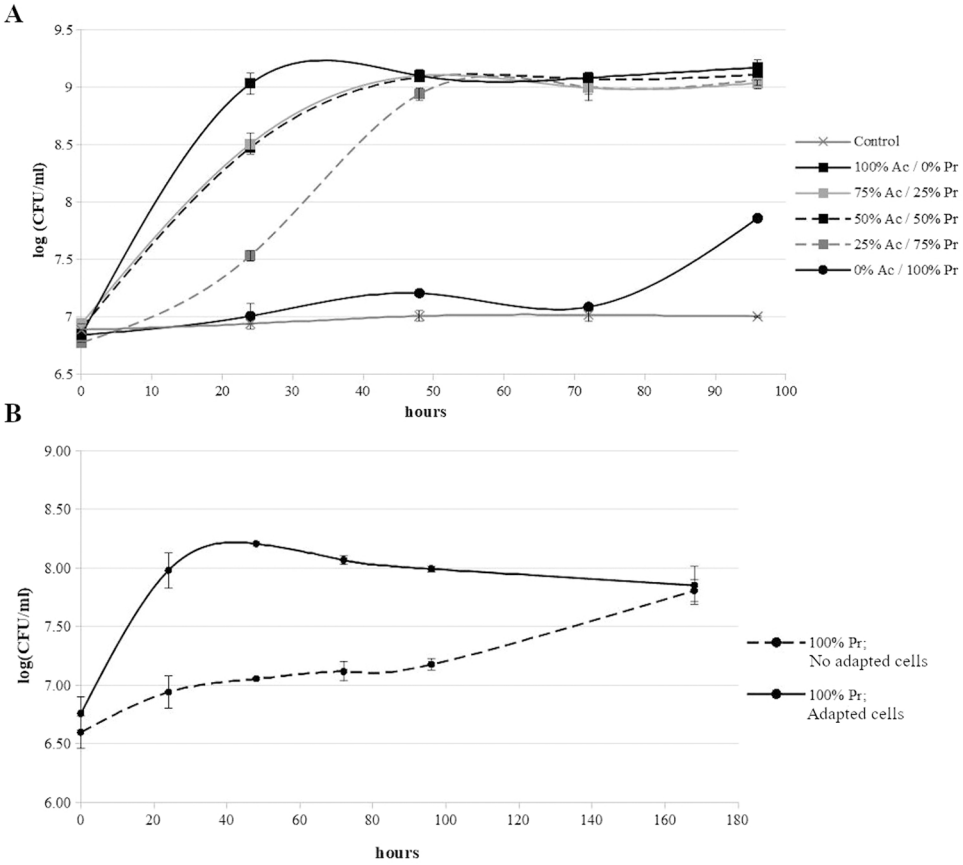

Fig. 1. (A) Time courses of microbial growth by *Thauera* sp. Sel9 performed in mineral medium Brunner with different ratios of acetate (Ac) and propionate (Pr) added as sole sources of carbon and energy. **(B)** Growth curves of Sel9 carried out in mineral medium Brunner supplied with propionate as carbon substrate, with or without adaptation previously performed in TSB medium with added acetate and propionate (1:1). The total concentration of the carbon sources was fixed at 1 g COD/L; COD:N was 9.4 and pH 7.5. Each curve shows means based on the results of three experiments.

Table 2
VFA concentrations at the beginning and after 96 h growth of *Thauera* sp. Sel9 using acetate (Ac) and propionate (Pr) as carbon sources.

	0 h (mg/mL)		96 h (mg/mL)	
	Ac	Pr	Ac	Pr
Ac	1276 ± 27	0	13 ± 2	0
Ac/Pr (3: 1)	887 ± 20	370 ± 10	13 ± 0	0
Ac/Pr (1: 1)	451 ± 15	645 ± 11	10 ± 1	0
Ac/Pr (1: 3)	295 ± 6	799 ± 16	12 ± 1	0
Pr	0	1455 ± 29	0	1164 ± 133

substrate jointly with unbalanced nutritional conditions, mainly due to low content of nitrogen and/or phosphorous sources [9]. PHAs can be produced by isolated bacterial strains using expensive carbon sources (e.g. glucose or saccharose) which contribute up to 40 % of the total cost of production [10]. Alternatively, they can be produced by selected mixed microbial cultures (MMCs) from biobased volatile fatty acids (VFAs) derived from the acidogenic fermentation of low-cost organic wastes [11,12]. Three main genera are found to be involved in PHA production in MMCs, *Azoarcus*, *Paracoccus* and *Thauera* [13,14]. The relative abundance of these genera depends on many factors such as sludge retention time (SRT) and carbon sources [13,14]. *Azoarcus* and *Thauera*

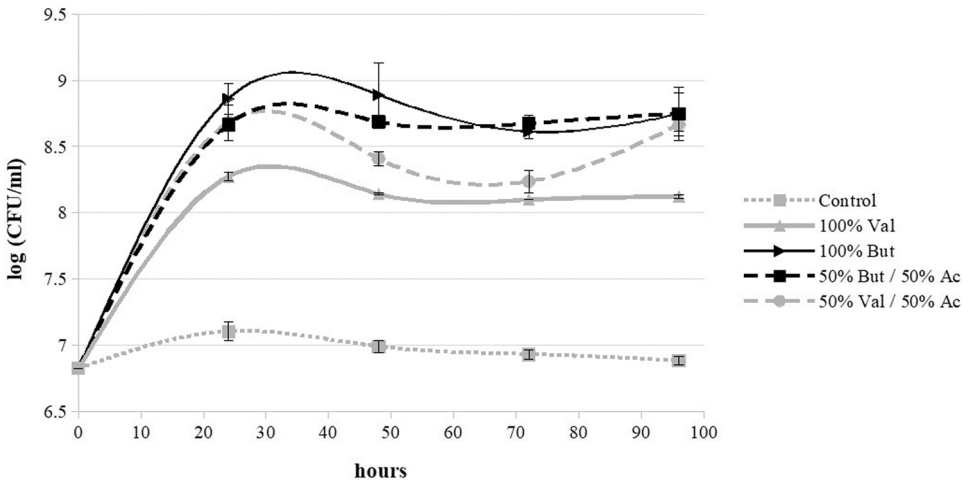

Fig. 2. Time courses of microbial growth by *Thauera* sp. Sel9 carried out in mineral medium Brunner added with butyrate (But) and valerate (Val) as sole sources of carbon and energy and mixed 1:1 with acetate (Ac). The total concentration of the carbon sources was fixed at 1 g COD/L; COD:N was 9.4 and pH 7.5. Each curve shows means based on the results of three experiments.

Table 3
VFA concentrations at the beginning and after 96 h growth of *Thauera* sp. Sel9 using butyrate (But) and valerate (Val) as sole carbon sources or mixed 1:1 with acetate (Ac).

	0 h (mg/mL)			96 h (mg/mL)		
	Ac	But	Val	Ac	But	Val
But	–	1257 ± 76	–	–	140 ± 9	–
Val	–	–	1123 ± 20	–	–	183 ± 9
Ac/But (1:1)	566 ± 15	536 ± 47	–	1 ± 0	152 ± 13	–
Ac/Val (1:1)	507 ± 12	–	517 ± 10	10 ± 2	–	0

were identified as the dominant genera in the presence of acetate and butyrate, whereas *Paracoccus* spp. may grow on a wider range of substrates [13]. *Thauera* genus can reach > 50 % of the total MMCs and is able to synthesize PHBHV with different 3HV unit content by using different ratio of acetic and propionic acid [14,15].

However, it appears that no strain belonging to the *Thauera* genus has so far been isolated from selected MMCs for PHA production. Therefore, the main aim of this work was to isolate a bacterial strain belonging to *Thauera* genus from an MMC for accumulation of PHAs using different VFAs as substrate. The isolated strain – namely *Thauera* sp. Sel9 – was further characterized to evaluate either the growth or PHA accumulation performance by using different VFAs as substrates. Specifically (i) the growth of this strain was investigated by using different COD:N ratios and VFAs acetate, propionate, butyrate and valerate as carbon and energy sources; (ii) the PHA accumulation of Sel9 was evaluated using different COD:N ratios and the above VFAs as carbon and energy sources; (iii) the complete genome of Sel9 was sequenced to identify the genes involved in PHA production, regulation and degradation.

Table 4
VFA concentrations at the beginning and after 96 h growth of *Thauera* sp. Sel9 at different COD:N ratios in presence of acetate (Ac) and propionate (Pr) (1:1) as carbon sources.

	0 h (mg/mL)		96 h (mg/mL)	
COD: N	Ac	Pr	Ac	Pr
4.7	581 ± 14	664 ± 7	14 ± 1	1 ± 0
9.4	654 ± 19	751 ± 8	17 ± 2	0
18.8	642 ± 11	745 ± 16	9 ± 1	0
37.6	500 ± 5	565 ± 7	13 ± 2	0
75.2	508 ± 21	576 ± 9	364 ± 8	1 ± 0
150.4	596 ± 9	694 ± 8	436 ± 49	262 ± 7
300.8	640 ± 4	751 ± 6	418 ± 132	299 ± 144
No ammonia	660 ± 10	757 ± 7	585 ± 1	625 ± 177

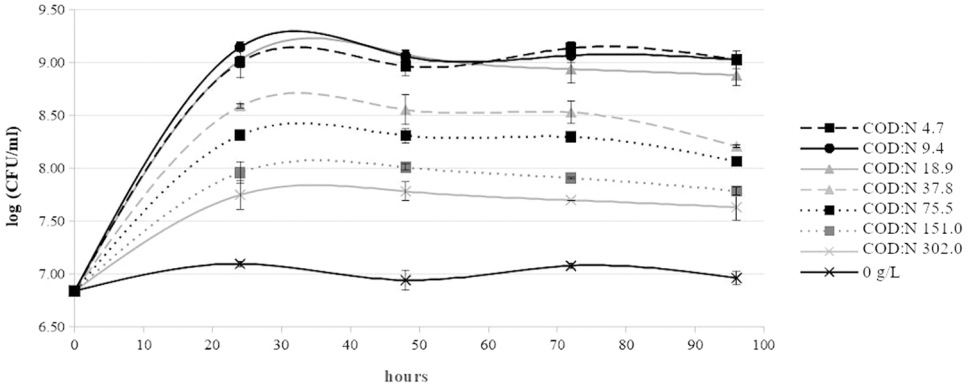

Fig. 3. Timecourses of microbial growth by *Thauera* sp. Sel9 at different COD:N ratios performed in mineral medium Brunner with added acetate and propionate (1:1) as carbon sources. Bacterial growth was also monitored in the absence of nitrogen source (0 g/L) as a control. The total concentration of the carbon sources was fixed at 1 g COD/L and pH 7.5. Each curve shows means based on the results of three experiments.

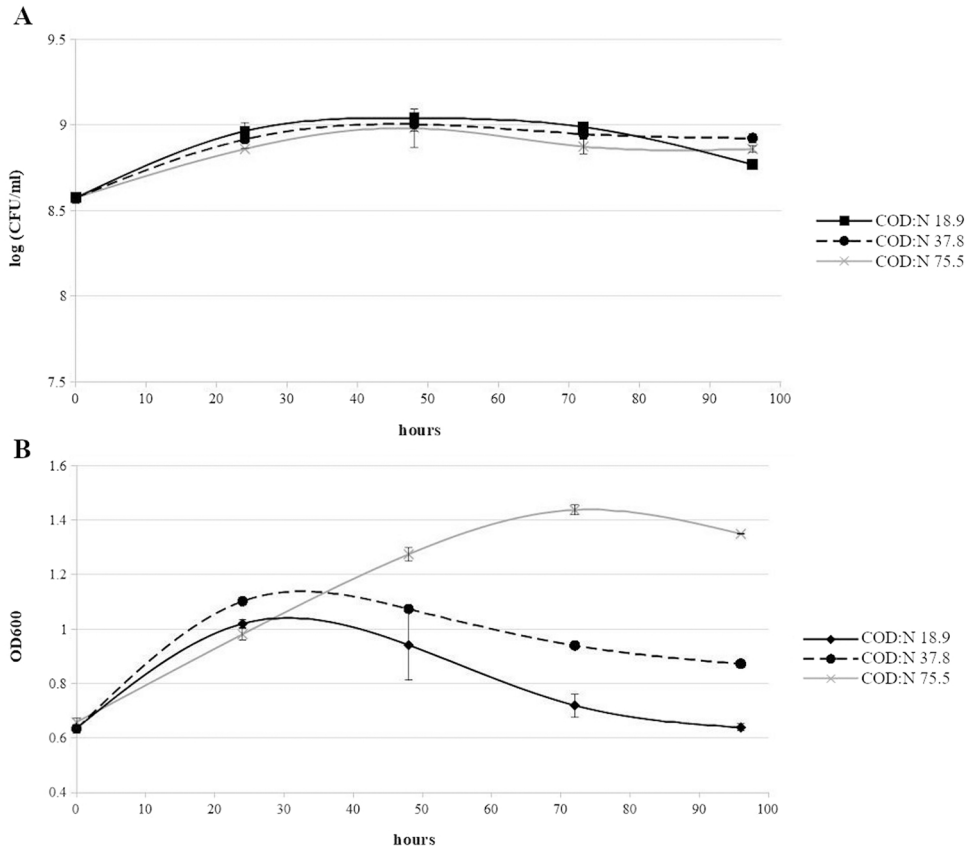

Fig. 4. Profiles of *Thauera* sp. Sel9 determined by both **(A)** CFU counts and **(B)** OD_{600} during PHA accumulation phase at different COD:N ratios. The experiment was conducted in mineral medium Brunner with added acetate (1 g COD/L). Final pH was 7.5. Each curve shows means based on the results of three experiments.

Table 5
Dry mass of and relative fluorescence intensity (AU) of *Thauera* sp. Sel9 at the end of the PHA accumulation phase using different COD:N ratios in the presence of acetate. Each value was based on the results of three experiments. Relative fluorescence intensity is expressed as Arbitrary Units.

	Dry biomass (g/L)	Relative fluorescence Intensity (AU)
COD:N 18.9	0.26 ± 0.2	5225 ± 115
COD:N 37.8	0.33 ± 0.1	5540 ± 290
COD:N 75.5	0.45 ± 0.3	8060 ± 385

Materials and methods

Isolation of the strain Thauera sp. Sel9

The strain Sel9 was isolated from a sequencing batch reactor (S-SBR) for the selection of PHA storing biomass from MMCs. The selection strategy adopted in the S-SBR consisted of feast and famine cycles under both aerobic and anoxic conditions [14,16] (see Supplementary material Tab. S1). The strain was screened for its capability to produce PHAs by Nile Red screen methodology [17] and further identified by 16S rRNA gene analysis [18]. The 16S rRNA gene (1392 bp) was amplified using fD1 and rp2 primers [19], sequenced by Eurofins Genomics (Ebersberg, Germany), and further analyzed using EzBioCloud [20]. The 16S rRNA gene was deposited in GenBank with Accession no. OP279920. The strain was routinely grown in Tryptone Soya Broth (TSB) or Agar (TSA) (Oxoid, Basingstoke, UK).

Genome analysis

The genomic DNA of *Thauera* sp. Sel9 was obtained using the FastDNA Spin kit for soil (MP Biomedicals, Irvine, CA, USA) with modification: the cells lysis step was replaced by lysozyme (Merck, Kenilworth, NJ, USA) (20 mg/mL) followed by a treatment with the Digestion Solution as described in the instruction manual (Wizard SV Genomic DNA Purification System, Promega; Madison, WI, USA).

The draft genome of *Thauera* sp. Sel9 was sequenced and assembled by IGATech (Udine, Italy). 'Celero™ DNA-Seq' kit (NuGEN; San Carlos, CA, USA) was used for library preparation following the manufacturer's instructions. Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and quality tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Santa Clara, CA, USA). The resulting libraries were then sequenced on Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) in paired-end 150 bp mode. Approximately 9 million reads per library were produced. Assembled contigs were polished with the Illumina reads, aligning them to the assembled sequence with BWA mem v0.7.17 [21] and calling homozygous SNPs and indels with pilon v1.23 [22]. A consensus sequence was created with bcftools v1.9 [23]. The genome of *Thauera* sp. Sel9 was submitted to GenBank, Accession no. JANUXN000000000. Gene annotation was performed by NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) [24] and the homolog proteins were found by BlastP program [25].

Growth curves using synthetic VFAs as carbon source

All the chemical compounds were purchased from Oxoid (Basingstoke, UK). A pre-inoculum of *Thauera* sp. Sel9 grown in TSB (unless otherwise noted) at 27 °C for 48 h on an orbital shaker at 200 rpm was

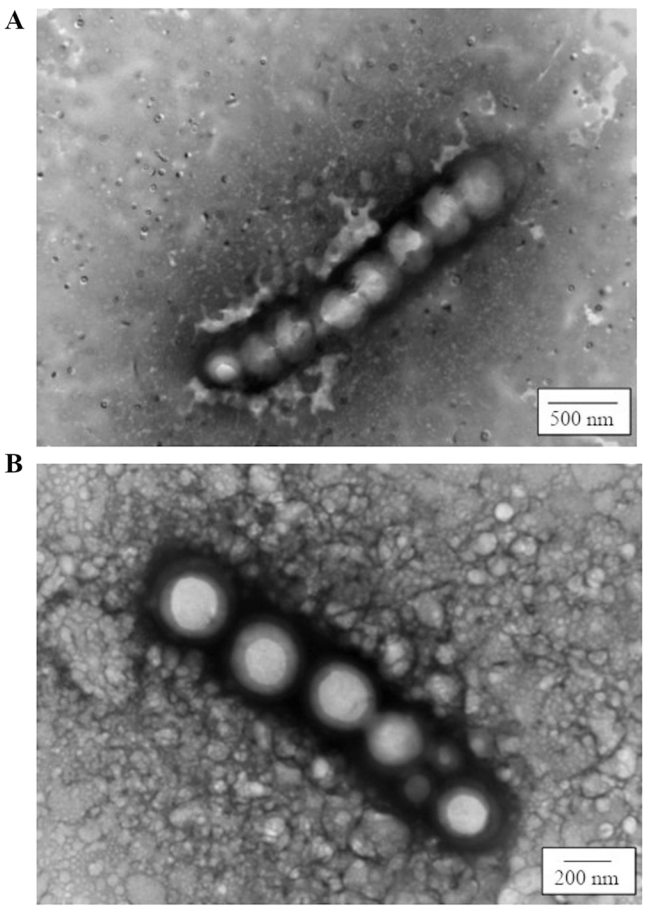

Fig. 5. PHA granules observed by TEM analysis in *Thauera* sp. Sel9 at the end of PHA accumulation phase. After the growth phase, the cells were incubated at 27 °C for 96 h in mineral medium Brunner with added acetate (1 g COD:L). COD:N was fixed at 75.5 and pH 7.5. The image magnifications were (A) 11,000X and (B) 18,000X.

centrifuged at 4500 *g* for 15 min at 4 °C and washed twice in physiological solution. The suspension was transferred in 25 mL, final OD_{600} of 0.01, of mineral medium Brunner (Medium 457, DSMZ) with some modifications: 2.44 g/L Na_2HPO_4, 1.52 g/L, KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $CaCl_2{\cdot}2H_2O$. Trace elements were replaced with the Wolfe and Vitaminic solution used in the Defined Medium (DM) [26]. Acetate (Ac), propionate (Pr), butyrate (But) and valerate (Val) were used as sources of carbon and energy, with $(NH_4)_2SO_4$ as nitrogen source. All carbon source ratios are expressed as COD:COD. The final pH was 7.5.

The composition and concentration of carbon and nitrogen were fixed for each trial as follows:

- Growth test 1. Different ratios of acetate and propionate were used: Ac, Pr, Ac:Pr (1:1), Ac:Pr (3:1) and Ac:Pr (1:3); the total concentration of the carbon source was fixed at 1 g COD/L; COD:N was at 9.4.
- Growth test 2. Two different growth media were used for the preparation of *Thauera* sp. Sel9 pre-inocula: (i) TSB and (ii) TSB supplied with Ac:Pr (1:1; 1 g COD/L); the growth curves were performed in Pr (1 g COD/L); COD:N was at 9.4.
- Growth test 3. Butyrate and valerate were tested both with and without acetate: But, Val, Ac:But (1:1) and Ac:Val (1:1); the total concentration of the carbon source was fixed at 1 g COD/L; the COD:N was at 9.4.
- Growth test 4. The test aimed to identify the most appropriate COD:N ratio for the growth of *Thauera* sp. Sel9 using Ac:Pr (1:1, 1 g COD/L) as carbon source. The COD:N ratio was fixed at: 4.7, 9.4, 18.9, 37.8, 75.5, 151 and 302. The growth of Sel9 without nitrogen source was monitored as a control.

Sel9 was incubated at 27 °C, on an orbital shaker at 200 rpm. The cell concentration was determined by Colony Forming Unit (CFUs/mL). Data from three independent experiments were collected.

PHA accumulation using synthetic VFAs as sources of carbon

A pre-inoculum of *Thauera* sp. Sel9 grown in TSB at 27 °C for 48 h (200 rpm) was centrifuged at 4500 *g* for 15 min at 4 °C and washed twice in physiological solution. Thereafter, the cell suspension was transferred so as to obtain a final OD_{600} of 0.01 in 100 mL of Brunner medium supplied with Ac:Pr (1:1, 1 g COD/L, COD:N 9.4, pH 7.5) and the cells were incubated for 72 h at 27 °C at 200 rpm. The culture was centrifuged at 4500 *g* for 15 min at 4 °C and washed twice in physiological solution. The cells were transferred in 100 mL of fresh Brunner medium with the following carbon and nitrogen compositions:

PHA accumulation, test 1. The test aimed to identify the most suitable COD:N ratio for the accumulation of PHAs: acetate was used as substrate (1 g COD/L); three different COD:N ratios were tested, namely 18.9, 37.8 and 75.5. Final pH was 7.5.

PHA accumulation, test 2. Different VFAs were used as substrate for the PHA accumulation: Ac, Pr, But and Val (1 g COD/L); the COD:N was fixed at 75.5. Final pH was 7.5.

The cells were thus incubated at 27 °C on an orbital shaker at 200 rpm and monitored every 24 h through the measurement of both OD_{600} and CFUs/mL. Each experiment was performed in triplicate.

Nile Red staining and fluorescence spectroscopy

The fluorescence spectroscopy analysis was performed during PHA accumulation, test 1 to determinate the most appropriate COD:N ratio for PHA accumulation. Nile Red staining of the cells was performed according to [27]. The cell-free medium, *Escherichia coli* DH5α cells and unstained *Thauera* sp. Sel9 were also stained and analyzed as controls. Fluorescence spectra were recorded using a Perkin–Elmer FL 6500 luminescence spectrophotometer (Perkin-Elmer, Waltham, MA, USA), equipped with Spectrum FL software (version 1.3.0.798). Total luminescence spectra, in the form of excitation–emission matrices (EEM, or 3D map), were obtained by setting the emission (Em) wavelength in the range 550–750 nm, while the excitation (Ex) wavelength was increased sequentially from 400 to 650 nm by 5 nm steps. All spectra were recorded using the same instrument conditions: Em and Ex slits were set at a 5 nm band width, and a scan speed of 1200 nm min^{-1} was selected for both monochromators. Relative fluorescence intensity (RFI) is expressed as Arbitrary Units (AU).

Transmission Electronic Microscopy (TEM)

The imaging of *Thauera* sp. Sel9 cells was performed using a Hitachi H-7650 120 kV transmission electron microscope (Hitachi, Tokyo, Japan). Aliquots (500 µL) of bacterial liquid culture grown with acetate (COD:N was fixed at 75.5) was recovered after 96 h of incubation and centrifuged at 5000 *g* for 10 min at 4 °C. The cell pellets were diluted in 10 µL of ddH_2O reaching a final CFUs/mL of 1×10^4, deposited on CF300-Cu-Carbon Film Copper grids (Società Italiana Chimici, Rome, Italy), and air dried for 24 h prior the TEM observation.

Chemical characterization

Mixed liquor suspended solids (MLSSs) and soluble COD (sCOD) were measured according to standard methods [28,29]. The concentration of each type of VFA was determined by ion chromatography through a Dionex ICS-1100 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with IonPac ICE-AS1 as the column, as described in [16].

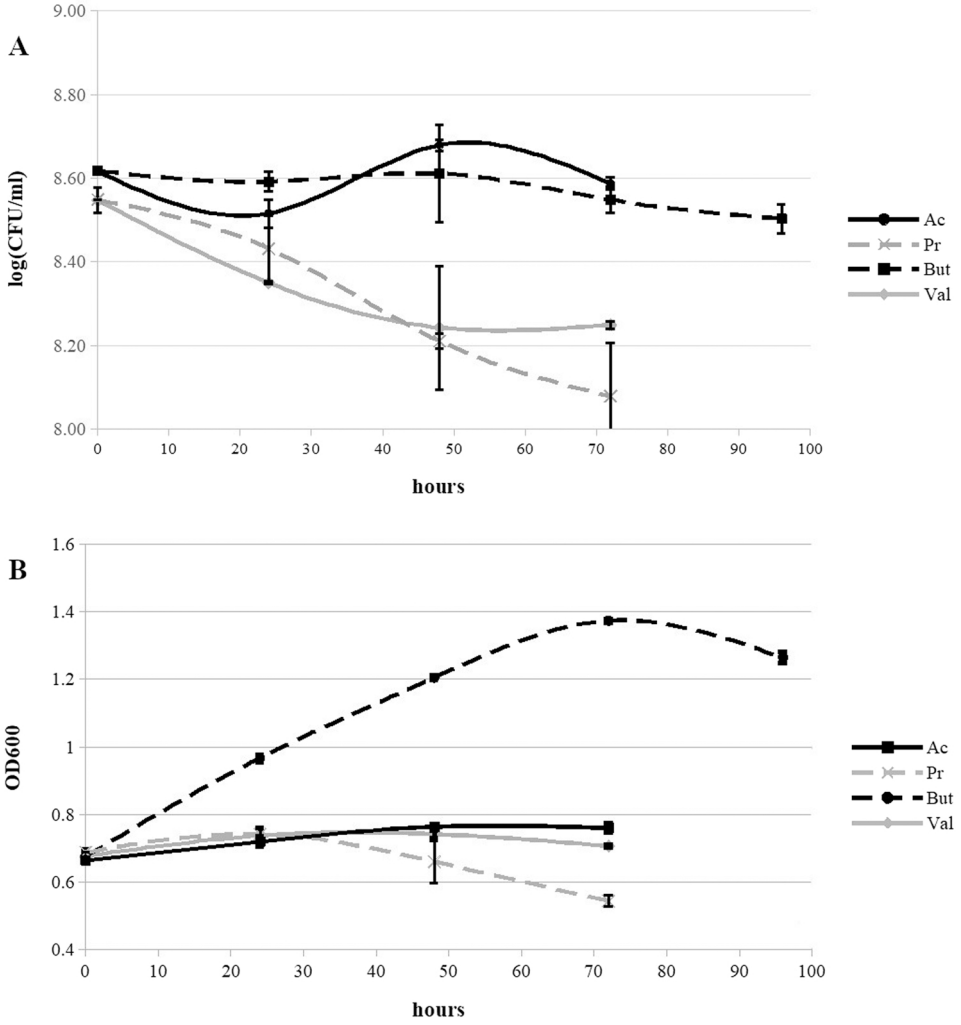

Fig. 6. Profiles of *Thauera* sp. Sel9 determined by both **(A)** CFUs counts and **(B)** OD_{600} during PHA accumulation phase. The experiment was conducted in mineral medium Brunner with added acetate (Ac), propionate (Pr), butyrate (But) or valerate (Val) as sole carbon sources (1 g COD/L). COD:N was 75.5 and pH 7.5. Each curve shows means based on the results of three experiments.

Table 6
PHA accumulation (% cell dry mass) and 3HB:3HV ratios by *Thauera* sp. Sel9 at the beginning (Post-Growth) and at the end of the PHA accumulation phase using acetate (Ac), propionate (Pr), butyrate (But) and valerate (Val) as sole source of carbon and energy. Each value represents the average and the standard deviation of three experiments.

	PHAs (% cell dry mass)	3HB: 3HV
Post-Growth	3.55 ± 2.87	1.15 ± 0.19
Ac	49.31 ± 0.70	3.56 ± 0.02
Pr	18.06 ± 3.81	0.76 ± 0.02
But	60.12 ± 2.60	12.30 ± 0.82
Val	37.31 ± 0.43	0.93 ± 0.03

Ammoniacal nitrogen (NH_4^+) was determined by the kit Ammonium LCK303 (Hach Lange GMBH, Loveland, CO, USA).

The PHA content, evaluated during the PHA accumulation tests, was determined as follows. The Sel9 cells were pelleted by centrifugation and freeze-dried. The biomass (10 mg) was treated with 1 mL of chloroform containing 0.5 % of boric acid *(w/v)* and 2 mL of methanol containing 3 % of sulfuric acid. Thereafter, the samples were digested for 4 h at 100 °C. After cooling, 1 mL of distilled water was added and vortexed for 15 min. The quantification of the PHAs was performed by the injection of 1 μL of organic phase into the gas chromatograph as described in [30].

Results and discussion

Phylogenetic analysis and identification of genes involved in PHA synthesis, regulation and degradation

The analysis of the 16S rRNA gene sequence showed that strain *Thauera* sp. Sel9 shares high sequence similarity with *T. butanivorans* NBRC 103042T (98.99 %) and *T. linaloolentis* DSM 12138T (98.49 %). All the other type strains evidenced a sequence similarity < 97 %. The *Thauera* genus includes 15 validly published species isolated from different environmental samples such as a hot spring and a microbial fuel cell [31,32] and further described for their catabolism versatility [33–35], humus reducing property [31] or for their peculiar anaerobic respiration [36]. Moreover, *Thauera* is a well-known genus involved in the conversion of VFAs to PHAs in MMCs [13,14,37]. Although few efforts have been made to use different VFAs as substrates for synthesis of PHAs from bacterial isolates [38,39], this study reports for the first time the description of a *Thauera* sp. strain (Sel9) isolated from an MMC. The high similarity of Sel9 to *T. butanivorans* and *T. linaloolentis* is consistent with the PCR-DGGE (denaturing gradient gel electrophoresis) results performed in the selection S-SBR from which Sel9 was isolated. Bands

extracted from DGGE gel were in fact ascribable to these species [14]. *T. butanivorans* NBRC 103042T and *T. linaloolentis* DSM 12138T show facultatively anaerobic chemoorganotrophic metabolism with the ability to utilize nitrate as a terminal electron acceptor and many VFAs such as acetate, propionate and butyrate as sole carbon source [35,40]. Descriptions regarding PHA accumulation in strains belonging to these species were not found in the literature.

The assembled genome sequence consists of 4,553,446 nt with a DNA GC content of 66 %. The homologous genes and relative proteins involved in PHA synthesis, the gene for phasin and that for its regulation and PHA degradation were identified (Table 1). Further BlastP confirmed that the proteins found in Sel9 may be ascribed to acetyl-CoA C-acetyltransferase (PhaA), acetoacetyl-CoA reductase and 3-ketoacyl-ACP reductase (PhaB), poly(R)-hydroxyalkanoic acid synthase (PhaC), phasin (PhaP), polyhydroxyalkanoate synthesis repressor (PhaR) and PHB depolymerase/esterase (PhaZ). Amino acid identity ranged from 95.04 % to 100 % with the homologous proteins of *T. butanivorans* NBRC 103042T (Table1). Homolog genes were already found in different beta-proteobacteria strains including *Thauera aminoaromatica* MZ1T [5]. The genome annotation of Sel9 is a fundamental step for future metabolic engineering purposes aimed at optimization of the PHA metabolic pathways [41,42]. One of the most prominent approaches may be the repression of the PHA degradation pathway through the deletion of endogenous PHA depolymerase (*phaZ*) [42].

Growth test of Thauera sp. Sel9

Effect of different VFAs on the growth of Sel9

The first experiment aimed to evaluate the ability of the Sel9 strain to use acetate and/or propionate – the shortest VFAs harboring C2 and C3, respectively – at different ratios as sole source of carbon and energy. The results showed that Sel9 reached about 9 log (CFUs/mL) after 72 h of incubation when acetate – either as single carbon source or in co-presence of propionate - was supplied in the growth medium. However, both acetate and propionate were here totally consumed after 96 h. In contrast, Sel9 showed a lag period of 72 h in the presence of 100 % propionate, and a consumption of this VFA of about 190 g COD/L after 96 h of incubation (Fig. 1 A; Table 2). On the other hand, it was observed that *Thauera* sp. Sel9 could reach the stationary phase – about 8 log (CFUs/mL) – on propionate without showing lag phase after 48 h when adapted with acetate/propionate (1:1) (Fig. 1B).

The growth of Sel9 on butyrate (C4) and valerate (C5) was investigated (Fig. 2). Data showed that when butyrate was supplied in the medium, Sel9 reached a cell concentration of about 9 log (CFUs/mL) either in co-presence or absence of acetate. On the other hand, the same cell concentration of Sel9 was obtained with valerate only in presence of 50 % of acetate. The chemical analysis revealed that all the VFAs supplied were consumed after 96 h of incubation (Table 3).

The results thus far achieved showed that acetate and butyrate were the most suitable VFAs to support Sel9 growth. Otherwise, propionate was consumed only after a 72 h lag phase, or in co-presence of acetate, or after cell adaptation with a mixture of acetate and propionate. It is well known that VFA composition is an important parameter affecting the bacterial community in MMCs. In mixed cultures *Thauera* was shown to prefer acetate and butyrate, whereas *Azoarcus* and *Paracoccus* were dominant in presence of propionate [13,14,43].

Effect of the COD:N ratio on the growth of Sel9

The growth curve of *Thauera* sp. Sel9 on acetate and propionate (1:1; 1 g COD/L) was monitored under different COD:N ratios: 4.7, 9.4, 18.9, 37.8, 75.5, 151 and 302 (Fig. 3). The results showed that the higher cell concentration – 9 log (CFUs/mL) after 48 h from the inoculum – was obtained by using COD:N 4.7, 9.4 and 18.9. On the other hand, Sel9 reached a cell concentration of about 8.2, 8.1, 7.7 and 7.6 log (CFUs/mL) with COD:N of 37.8, 75.5, 151 and 302 respectively. No growth was observed in the absence of N in the medium. In accord with the growth dynamics, chemical analysis showed a complete depletion of VFAs at COD:N ratios of 4.7, 9.4 and 18.9. In contrast, the concentration of residual VFAs at the end of the experiment progressively increased when the COD:N was > 37.8. (Table 4).

Accumulation of PHAs by Thauera sp. Sel9

Effect of different COD:N ratios on PHA accumulation

The production of PHAs by *Thauera* sp. Sel9 was investigated under 3 different COD:N ratios (18.8, 37.8 and 75.5 COD:N). The CFU count evidenced only a limited growth of Sel9 without significant differences between the different COD:N ratios, but the OD_{600} was clearly higher in the culture with COD:N 75.5 (Fig. 4). This result may suggest an increase in the total biomass of the culture despite the same number of living cells being retained. In fact, the dry biomass concentrations (calculated as MLSS) were 0.26 ± 0.2, 0.33 ± 0.1 and 0.45 ± 0.3 g/L operating at COD:N ratios 18.8, 37.8 and 75.5 respectively (Table 5).

The measurement of the RFI of Nile Red stained cells is a rapid and affective technique to determine PHA accumulation [27]. Firstly, a 3D contour map was obtained for *Thauera* sp. Sel9 to identify the most suitable wavelengths which can be used in subsequent tests. The stained cells were scanned at Ex wavelengths from 450 to 600 nm, and Em wavelengths from 550 to 750 nm. The Ex/Em wavelength pairs at the maximum RFI ($EEWP_{max}$; nm/nm) was 545 Ex/625 Em (see Supplementary material Fig. S1). On the other hand, no fluorophore was observed in all the controls: stained cell-free medium, *E. coli* DH5α cells, as well as unstained *Thauera* sp Sel9. Further fluorescence analysis showed an RFI at the $EEWP_{max}$ of 5225 ± 115, 5540 ± 290 and 8060 ± 385 AU for *Thauera* sp. Sel9 cells grown on COD:N 18.8, 37.8 and 75.5, respectively (Table 5). The presence of granules of PHAs was further detected by TEM which indicated multiple white spheres within the bacterial cells (Fig. 5).

It is well reported that the C:N ratio is an important parameter for PHA accumulation [44]. Overall, a lower C:N ratio is beneficial for cell growth while higher C:N ratios (N-limiting condition) promote PHA accumulation [45]. The data reported here showed two different optimal COD:N ratios: 4.7–18.9 for the bacterial growth and 75.5 for PHA accumulation. The evaluation of the most suitable C:N ratio for PHA accumulation was carried out by fluorescence spectroscopy analysis. This method is fast, accurate, non-destructive and requires low sample volumes to estimate PHAs in bacterial cells [27]. Based on 3D spectra, Ex and Em wavelengths of 545 and 625 nm, respectively, were used to measure the RFI of Sel9 grown on acetate. It is already known that the range of excitation (520–550 nm) and emission (590–630 nm) wavelengths can be used for all types of PHAs [27]. The same authors observed that the range of emission wavelength varied slightly among different polymers: the 605–630 range may be related to the polymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Several efforts were made to identify the most suitable COD:N in MMCs. For instance, it is reported that the highest PHB level (about 70 wt% of dry cell mass) was obtained at C:N 40 [46], whereas an optimal ratio of 100 is described [44]. Moreover, several studies stated different C:N ratios for PHA accumulation by pure cultures; for instance, a value of about 35 was obtained for *Comamonas* sp. EB172, *Cupriavidus necator* H16 and *Haloferax mediterranei* [47–49].

Accumulation of PHAs using different synthetic VFAs

The previous results showed that a COD:N of 75.5 was the most suitable culture condition for the production of PHAs by *Thauera* sp. Sel9. Therefore, this ratio was utilized in combination with different VFAs as carbon sources: acetate, propionate, butyrate and valerate. Biomass production measured through the CFU count did not change significantly in the presence of acetate and butyrate. Nevertheless, when propionate and valerate were supplied to the medium, a significant reduction in living cells was observed (Fig. 6A). In contrast, the turbidity of the culture, determined as OD_{600}, showed the highest value in the

presence of butyrate (~ 1.40 OD_{600}) followed by acetate (~ 0.75 OD_{600}), valerate (~ 0.7 OD_{600}) and propionate (~ 0.55 OD_{600}) (Fig. 6B). The concentration of PHAs in the cells and 3HB:3HV ratio were determined by gas-chromatography. The results indicated a very low amount of PHAs after the growth phase (3.55 ± 2.87 %), which corresponded at the beginning of the accumulation phase. Thus, a PHA content of 49.31 ± 0.7 %, 18.06 ± 3.81 %, 60.12 ± 2.60 %, 37.31 ± 0.43 % *(w/w)* of *Thauera* sp. Sel9 grown on acetate, propionate, butyrate and valerate, respectively, was determined. Furthermore, the 3HB:3HV ratios reflected the growth substrate: 3.56 ± 0.02 with acetate, 0.76 ± 0.02 with propionate, 12.30 ± 0.82 with butyrate and 0.93 ± 0.03 with valerate (Table 6).

In terms of PHA storage, about 60 % and 50 % *(w/w)* of PHAs were obtained in Sel9 by using butyric and acetic acids respectively. A few studies have reported PHA production by an isolated bacterial strain using VFAs as sole sources of carbon and energy. *Pseudomonas oleovorans* reached a maximum concentration of 26 % PHA content with acetic acid as substrate [39]; moreover, the highest accumulation of PHAs (57 ± 5 %) in *Pseudomonas pseudoflava* was achieved with 20 g/L of VFAs. *Thauera aminoaromatica* MZ1T reached about 30 % of PHA content in cell dry mass (wt) by using 10 g/L of acetate (C:N ratio of 10:1 mol/mol) [37], whereas it was reported that a more continuous VFA feeding regime can lead to a 75 % (*w/w*) of PHA in *Cupriavidus necator* DSMZ 545 [38]. On the other hand, it is important to underline that more investigations should be performed on *Thauera* sp. Sel9 to maximize PHA productivity, considering different factors that can affect PHA synthesis, such as C:P ratio, pH control, initial VFA concentration and composition, temperature, duration of the PHA accumulation, and fed-batch vs continuous mode alimentation [38,50]. Moreover, to reduce production costs, the use of inexpensive VFA-rich substrates derived from fermented organic waste material should be taken into account [12,50]. The results showed that the 3HB:3HV ratio strongly depends on carbon source: acetate, and especially butyrate, drove towards higher ratios, whereas the presence of propionate and valerate drastically reduced these values. This observation agrees with PHA metabolism [9]. It was previously reported that in MMCs the addition of propionic acid altered the PHA composition, increasing the 3HV content in PHBHV from 2.71 % (sole acetate) to 94 % (sole propionate) [15]. Moreover, it is reported that the consumption of odd-numbered VFAs was strictly correlated with 3HV synthesis [51]. The mechanical and physical properties are fundamental parameters for a biopolymer's use, and the possibility to regulate the 3HB:3HV ratio as here observed is a great advantage in order to diversify their future applications. For instance, due their slow release of toxic anti-cancer drugs, PHA microparticles may be used for drug delivery purposes. This low release rate is higher in the case of PHBHV copolymers than for the PHB [52].

Conclusions

Thauera is one of the main genera involved in PHA accumulation in MMCs fed by VFAs [14,15]. The strain Sel9 is the first *Thauera* strain isolated from an S-SBR for PHA production which is able to grow and accumulate PHAs with different 3HB:3HV ratios by using a wide range of VFAs as sole sources of carbon and energy. Moreover, the homologous genes involved in PHA synthesis, regulation and degradation are identified here. The results allow a better understanding of the role and the contribution of *Thauera* in MMCs. Moreover, it strongly suggests a possible exploitation of *Thauera* sp. Sel9 for a cost-effective and environmentally friendly synthesis of PHAs with the possibility of regulating the 3HB:3HV ratio by using VFAs as substrate. Future analysis will be carried out to maximize PHA yield using fermented-derived VFAs.

CRediT authorship contribution statement

MA designed and conducted the laboratory experiments, analyzed the data, drafted the manuscript. VS conducted the laboratory experiments. NF analyzed the data. CZ conducted the laboratory experiments and analyzed the data. SL designed the laboratory experiments and analyzed the data. All the Authors contributed to discussing the results and writing the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.09.004.

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