Biocatalysis and Agricultural Biotechnology 20 (2019) 101268

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Burkholderia glumae MA13: A newly isolated bacterial strain suitable for polyhydroxyalkanoate production from crude glycerol

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ARTICLE INFO

Keywords:
Biodiesel
Bioplastic
Burkholderia glumae
Glycerol
Polyhydroxyalkanoates

ABSTRACT

Burkholderia glumae MA13 was isolated from soil samples of Atlantic rain forest ecosystem as a new bacterial strain for polyhydroxyalkanoate (PHA) production from crude glycerol as sole carbon source. Among 107 glycerol consuming bacterial isolates, *B. glumae* MA13 was cultivated in shake flask experiments in order to verify its PHA production capability from crude glycerol besides waste cooking oil and sugarcane molasses free of pretreatment, showing intracellular poly(3-hydroxybutyrate) [P(3HB)] accumulation values of 51.3, 51.4 and 49% of cell dry weight (CDW), respectively. The addition of propionic or hexanoic acids to the media containing crude glycerol resulted in the copolymers poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] comprised of up to 32.1 mol% of 3HV monomer or poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] with a maximum of 0.3 mol% of 3HHx, respectively. Fed-batch cultivation performed in a 10 L-scale bioreactor showed a maximum P(3HB-*co*-3HV) production of 9.9 g/L, which was 65.1% of total biomass and consisted of 21.8 mol% of 3HV monomer, with a maximum volumetric productivity of 0.22 g/(L h). The 3HV conversion yield achieved 1.04 g/g after 48 h cultivation, which was 76.7% of the maximum theoretical yield. *B. glumae* MA13 has showed to be an adapted bacterial strain to the synthesis of bioplastics from biofuel by-products, and so from here it has been revealed as a promising PHA producer for an associated production set which has been considered as a prominent and ecologically friendly alternative to petrochemical plastics and fuels.

1. Introduction

Currently, the industrialized world is highly dependent on fossil fuels as energy source for industrial processes and production of structural materials such as plastics, foams, coating and adhesives. However, the fossil fuels are a finite resource which becomes a global problem since our economy is still petroleum dependent. The present challenge is the replacement of conventional plastics from a non-sustainable source with a renewable one. Furthermore, petrochemical plastics not only take many decades to be decomposed in nature, but also produce toxins during degradation process (Suriyamongkol et al., 2007).

Environmentally degradable polymers are one of the possible solutions to substitute the petrochemical-based polymers. They are among the so-called biopolymers which can be classified into three groups based on their components and biodegradable characteristics. The first group consists of non-bio-based biopolymers with biodegradable or compostable properties such as polybutylene terephthalate copolymers (e.g., adipate, succinate, glutarate). Unlike, the second group is composed of bio-based resources but non-biodegradable properties. This group includes propanediol polymers and bioethanol-based linear low-density polyethylene. Finally, the third group refers to bio-based and biodegradable polymers such as polylactide, starch and cellulose-based materials, and polyhydroxyalkanoates (PHAs) (Avella et al., 2001; Chanprateep, 2010).

PHAs are microbial polyesters synthesized by many bacteria and archaea as intracellular storage compounds for energy reserve under culture conditions of carbon excess and nutrient limitation (e.g.,

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https://doi.org/10.1016/j.bcab.2019.101268
Received 12 April 2019; Received in revised form 19 July 2019; Accepted 24 July 2019

nitrogen, phosphorus, oxygen, magnesium) (Steinbüchel and Füchtenbusch, 1998; Koller, 2019). Recent research has demonstrated that PHAs are also involved in enhancing capabilities of stress resistance of prokaryotes with additional robustness and fitness of microbes under non-optimal growth conditions (Obruca et al., 2018). Among a large number of PHA-producing microorganisms and approximately 150 different types of monomer composition, poly(3-hydroxybutyrate) [P(3HB)] is the most common type and widely studied of all PHAs. The P(3HB) properties resemble those of petrochemical-based polypropylene (PP), which includes good processability on synthetic plastic equipment such as injection moulding, extrusion blow moulding and fibre spray-gun moulding, indicating that P(3HB) may be suitable for several applications as a partial substitute for non-biodegradable synthetic plastics (Steinbüchel and Füchtenbusch, 1998). Further, the P(3HB) properties can be improved by co-monomers incorporation such as 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx), and then resulting in the copolymers poly(3-hydroxubutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], respectively (Silva et al., 2000; Qiu et al., 2006; Lau et al., 2010).

Although PHAs represent environmental advantages to replace petrochemical polymers, the relatively expensive production prices of PHAs limit their use for general applications. Since 40–48% of total polymer costs are ascribed to raw materials from which the carbon sources can account up to 70–80%, alternative solutions have been proposed to implement waste carbon sources or industrial byproducts for bacterial cultivations aiming at reducing the PHA production costs (Choi and Lee, 1997; Verlinden et al., 2007; Cavalheiro et al., 2009; Koller et al., 2017; Kourmentza et al., 2017; Brigham and Riedel, 2019). Beside this challenge the biodiesel industries generate from the transesterification of vegetable oils and animal fats one of the promising alternative and renewable fuels, which has been developed in recent years. However, the biodiesel production has increased at a slower rate, whose main delay has been also attributed to its relatively high production cost (Yang et al., 2012).

The main byproduct from biodiesel production is crude glycerol which has a low value due to the presence of impurities (e.g., alcohols, salts and fatty acids). The refining of crude glycerol is economically counterproductive, especially for smaller scale producers who are vital to achieve the bioenergy goals. Once crude glycerol is becoming a waste product with an associated disposal cost, it is important to find environmentally friendly upgrades for this byproduct. Besides the search for value-added applications for crude glycerol, the biodiesel industries have also focused their efforts on utilizing low cost raw materials in order to reduce the biodiesel production costs. Waste cooking oil is available in large amounts whose management has posed a significant challenge, since the inappropriate disposal of this waste material is a threat to water and land resources. Therefore, the biodiesel manufacturers have encountered in this residue a low cost feedstock to produce biodiesel (Chhetri et al., 2008).

On the other hand, sugarcane molasses is a high sugar content byproduct from sugar biorefineries, which is directly involved in the bioethanol production, one of the most common and well established biofuels (de Paula et al., 2018). Therefore, due to high availability of low cost substrates related to biofuel industries, the implementation of biorefineries that coproduce value-added products along with biofuels has been proposed as a solution to support their industrial processes and market development, especially those derived from industrial byproducts and waste materials (Yazdani and Gonzalez, 2007; Cavalheiro et al., 2009; Ashby et al., 2011; Koller et al., 2017). Hence the conversion of biofuel byproducts into PHAs is economically interesting to enable the establishment of an environment-friendly and renewable outlet for petrochemical products based on biofuels and biopolymers. In this work, *Burkholderia glumae* was isolated from Atlantic ecosystem as a new and promising bacterial strain for PHA production using industrial byproducts as carbon source, with special focus on crude glycerol utilization.

2. Materials and methods

2.1. Bacterial isolation and characterization

Bacterial strains were isolated from soil samples of Atlantic rainforest in Ubatuba, São Paulo State, Brazil (23°34′58.53″S, 45°16′12.91″ W). Mineral salts medium (MSM) was used for isolation of glycerol consuming bacteria (Ramsay et al., 1990), at pH 7.0, containing (g/L): Na_2HPO_4, 3.5; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.01; Fe(III)NH_4-citrate, 0.06; and 1 mL trace elements solution containing (g/L): H_3BO_3, 0.3; $CoCl_2{\cdot}6H_2O$, 0.2; $ZnSO_4{\cdot}7H_2O$, 0.1; $MnCl_2{\cdot}4H_2O$, 0.03; $NaMoO_4{\cdot}2H_2O$, 0.03; $NiCl_2{\cdot}6H_2O$, 0.02; $CuSO_4{\cdot}5H_2O$, 0,01. 20 g/L pure glycerol and 1 g/L $(NH_4)_2SO_4$ were added to the culture media as carbon and nitrogen sources, respectively. The solid media were obtained by adding 20 g/L agar. The plates were incubated at 30 °C for up to 120 h. PHA-producing bacteria were screened in solid nitrogen limiting MSM containing 0.06 g/L $(NH_4)_2SO_4$ and 20 g/L pure glycerol. The plates were incubated at 30 °C and 37 °C for 120 h. PHA-producing strains were identified by dark blue color after Sudan Black B staining (Schlegel et al., 1970).

Morphological and biochemical characterization was performed for the most promising PHA-producing bacterial strain. The morphological characteristics were identified by bright-field microscopy after Gram staining. The biochemical characterization was performed using the API 20 NE kit (Biomerieux, France) to determine nitrate reduction; indole production; glucose fermentation; arginine dihydrolase, β-galactosidase and urease activities; hydrolysis of gelatin and esculin. Lipase, catalase and oxidase tests were also performed. Carbohydrate assimilation were determined using the API 50 CH kit (Biomerieux, France).

2.2. 16S rRNA gene and phylogenetic analyzes

Bacterial identification was performed by 16S rRNA gene analysis of the best PHA-producing bacterial isolate. The bacterial strain was cultivated in nutrient broth (NB: 5 g/L peptone, 3 g/L meat extract) for 24 h at 30 °C and 150 rpm. Cells were harvested by 15 min centrifugation at 10,000 *g* for DNA extraction. The 16S rRNA gene was amplified by PCR using the Pure Taq Ready-To Go PCR Beads with the universal primers for prokaryotes 27F (5′-AGAGTTTGATCA/CTGGCTCAG-3′) and 1492R (5′-ACGGT/CTACCTTGTTACGACT-T-3′). The PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK). BigDye Terminator v3.1 Cycle Sequencing kit was used to prepare the sequencing according to procedures provided by the manufacturer. The 16S rRNA gene sequencing was carried out on automatic capillary sequencer ABI 3500 (Applied Biosystems, USA). The consensus sequences were aligned with previously published sequences of bacterial strains using the BLASTn function available on the National Center for Biotechnology Information (NCBI) database. Phylogenetic and molecular evolutionary analyzes were conducted using the software MEGA 6.06 (Tamura et al., 2013).

2.3. Shake flask cultivations

The bacterial isolates tested in solid MSM and selected as PHA-producing strains after Sudan Black B staining were cultivated in shake flask experiments to confirm their PHA production capability. Bacterial strains from stock cultures were streaked on nutrient agar (NB plus 20 g/L agar) for 24 h at 30 °C, with subsequent transferring of bacterial colonies to 250 mL Erlenmeyer flasks containing 100 mL NB for 24 h, at 30 °C and 150 rpm. Aliquots of 3 mL from these cultivations were used to inoculate 250 mL Erlenmeyer flasks containing 100 mL MSM added to 20 g/L pure glycerol and 1 g/L $(NH_4)_2SO_4$, which were incubated for 72 h, at 30 °C and 150 rpm. *B. glumae* MA13 was also tested in MSM containing crude glycerol from Petrobras biodiesel plant (Candeias,

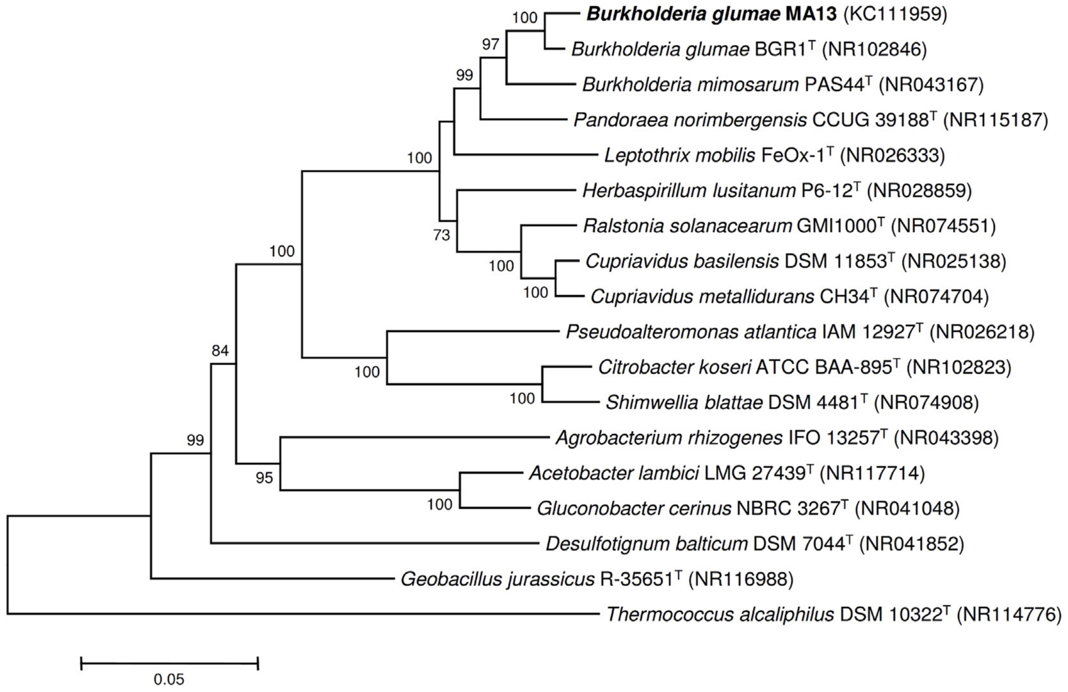

Fig. 1. Phylogenetic tree derived from neighbour-joining analysis showing phylogenetic position of *Burkholderia glumae* MA13 isolate based on 16S rRNA gene sequences comparison. Numbers above nodes represent bootstrap confidence values above 70% from 1000 resamplings. Bar scales indicate 0.05% sequence dissimilarity.

Brazil), which was composed of 74% glycerol, 4.4% hexane solubles, 18.5% water, 3% ash and < 0.5% methanol (w/w). Crude glycerol was added to the medium according to glycerol concentration which was adjusted from 10 to 50 g/L. Additionally, shake flask experiments were conducted to evaluate the potential of *B. glumae* to synthesize PHAs from waste cooking oil and sugarcane molasses compared to pure and crude glycerol carbon sources at 10 g/L. Waste cooking oil concentration was obtained after direct weighing whereas sugarcane molasses concentration was adjusted considering the amount of total sugars. Waste cooking oil was obtained from restaurants in Rio Claro, Brazil, and consisted of 15% palmitic acid, 4% stearic acid, 30% oleic acid, 43% linoleic acid, 4% linolenic acid and 4% other fatty acids (w/w). Sugarcane molasses was provided by São Domingos sugarcane biorefinery (Catanduva, Brazil), which was composed of 58.6% sucrose, 7.6% glucose, 8.3% fructose, 11% ash and 14.5% water (w/w). Moreover, kinetic experiments were carried out for 120 h in order to evaluate the PHA production after nitrogen limitation at intervals of 24 h. These cultivations were performed at 30 °C and 150 rpm in MSM containing 10 g/L crude glycerol as sole carbon source and plus propionic or hexanoic acids at 1 g/L, which were added to the culture media after 24 h cultivation as copolymer precursors in order to verify the ability of *B. glumae* to produce 3HV and 3HHx monomers, respectively. Copolymer precursors were added to the culture media after 24 h at low concentration when minimum biomass values were achieved, since they were also inhibitors of bacterial growth.

2.4. Bioreactor experiment

P(3HB-*co*-3HV) production from crude glycerol and propionic acid was performed in 10 L-scale bioreactor (Infors HT, Switzerland) under feed regimen following two steps of cultivation. Inoculum was prepared from transferring cell colonies to 50 mL NB medium for 12 h at 30 °C and 150 rpm, from which 30 mL were added to 500 mL MSM containing 10 g/L crude glycerol and 1 g/L $(NH_4)_2SO_4$ with subsequent incubation for 18 h at 30 °C and 150 rpm. In the first step, 500 mL inoculum was added to 4.5 L MSM containing 20 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$ and 2 mL trace elements solution. Second cultivation step started after 24 h cultivation under feed regimen of 200 g/L crude glycerol and propionic acid (10:1 w/w) to the culture broth at constant flow of 30 mL/h, which was kept up to 48 h cultivation. Fed-batch experiment was conducted at 30 °C and pH 7.0 maintained by automatic addition of 5M NaOH. Dissolved oxygen was kept at 20% saturated air by agitation (200–600 rpm) and aeration (0.5–2 vvm). Antifoam was added to cultivation broth by automatic sensors.

2.5. PHA determination

Bacterial cells were harvested by 15 min centrifugation at 10,600 g and 4 °C, washed twice, frozen and lyophilized. Biomass was determined gravimetrically as grams of cell dry weight (CDW) per liter of culture broth. Residual biomass was calculated subtracting PHA content from CDW. The PHA quantification and monomer composition were determined by gas chromatography (GC) of propyl esters. Samples of about 10 mg lyophilized cells were subjected to propanolysis reaction (Riis and Mai, 1988). Volumes of 1 μL organic phase containing the propyl esters were analyzed after split injection (1:25) by a flame-ionization detector (FID). PHA content was determined using Agilent 7890A GC System (Agilent Technologies, USA) with a HP-5 capillary column (5% phenyl methylpolysiloxane, 30 m × 0.25 mm x 0.25 μm, Agilent Technologies, USA). The temperatures of injection and detector were 250 and 300 °C, respectively. A temperature program was applied at 100 °C for 1 min, increasing 8 °C/min up to 185 °C, which was kept for 15 min. Helium was used as the carrier gas at a constant flow of 0.8 mL/min. Benzoic acid was used as internal standard. P(3HB-*co*-3HV) copolymer containing 14% 3HV (Aldrich, USA), mcl-PHAs produced by *Pseudomonas putida* ATCC 29,347 from fatty acids and *P. putida* IPT 046 from glucose were used as external standards. Monomer composition was confirmed by coupled GC-MS with quadrupole analyzer (14B/QP5050A Shimadzu, Japan). A volume of 0.4 μL organic phase was splitless injected in a BPX-5 capillary column (5% phenyl polysylphenylene-siloxane 30 m × 0.25 mm x 0.25 μm, SGE Analytical Science, Australia). The injector and detector temperatures were 280

Table 1
Biochemical and morphological characterization of *Burkholderia glumae* MA13.

Test	*Burkholderia glumae* MA13
Gram staining	–
Cell shape	Rod
Catalase	+
Oxidase	–
Nitrate reduction	+
Indole production (Tryptophane)	–
Glucose fermentation	–
Arginine dihydrolase	–
Urease	+
β-glucosidase (esculin)	+
Lipase	+
Gelatin	–
β-galactosidase	–
Glucose	+
Glycerol	+
Mannose	+
Manitol	+
N-acetyl-glucosamine	+
Potassium gluconate	+
Capric acid	+
Adipic acid	+
Malic acid	+
Citric acid	–
Phenylacetic acid	+
Adonitol	-
Arabinose	+
Arabitol	+
Cellobiose	-
Fructose	+
Fucose	+
Maltose	+
Melibiose	-
Raffinose	-
Rhamnose	-
Sorbitol	+
Sucrose	+
Trehalose	+
Xylitol	-
Ribose	+
Xylose	+
Inositol	+
Starch	-

Table 2
P(3HB) production by different bacterial isolates from Atlantic forest ecosystem. Mean values from shake flask experiments utilizing pure glycerol as sole carbon source.

Bacterial isolate	CDW (g/L)	P(3HB) (% CDW)	P(3HB) (g/L)
MA 03	1.80	16.20	0.29
MA 09	2.20	15.64	0.34
MA 13	4.20	46.27	1.94
MA 18	1.86	9.32	0.17
MA 82	1.28	0.53	0.01
MA 84	1.85	0.87	0.02

and 320 °C, respectively. The program temperature applied to the column started at 100 °C, increasing 8 °C/min up to 210 °C, which was kept for 15 min. Helium was used as carrier gas at a flow of 2 mL/min. PHAs were expressed as grams per liter, percentage of cell dry weight (% CDW) and molar percentage of constituent monomers (mol%). Polymer yield was calculated by dividing grams of polymer per grams of consumed carbon source.

2.6. Carbohydrate and nitrogen quantification

Glycerol, propionic acid, sucrose, glucose and fructose quantification were determined by HPLC apparatus (Shimadzu, Japan) with a Rezex ROA column (8% cross-linked sulfonated styrene-divinylbenzene; 300 mm × 7.8 mm; Phenomenex, USA). Samples of 20 µL were injected and eluted with 0,0025 M H_2SO_4 at a flow rate of 0.6 mL/min. The column oven temperature was kept at 65 °C. The UV/Vis detector was used to determine propionic acid concentrations whereas a RID-10A detector was used to determine glycerol, sucrose, glucose and fructose concentrations. Hexanoic acid was determined by GC (Agilent 7890A GC System, USA) with 0.2 µL supernatant portions injected into a FFAP column (30 m × 0.53 mm x 1.33 m; Agilent Technologies, USA). The injection and FID temperatures were 250 °C e 300 °C, respectively. Helium was used as carrier gas at 3 mL/min and the oven temperature program was applied at 130 °C for 5 min, increasing at 10 °C/min rate up to 185 °C. Glycerol, propionic acid, hexanoic acid, sucrose, glucose and fructose were used as external standards. The residual oil was determined after hexane extraction. 5 mL supernatant was added to 5 mL hexane and centrifuged at 10,600 *g* and 4 °C, for 15 min. 3 mL upper phase was transferred to tared vials with subsequent solvent evaporation for 24 h. The residual oil was determined gravimetrically as grams per liter of hexane solubles. Crude glycerol was determined according to its glycerol content, whereas sugarcane molasses was quantified considering the amount of total sugars (sum of sucrose, glucose and fructose concentrations). All standards and carbon sources were expressed as grams per liter. Nitrogen was determined by ammonium concentration using an ammonia ion selective electrode (Orion 9512BNWP, Thermo Scientific, USA).

3. Results and discussion

3.1. Identification of B. glumae MA13

B. glumae was identified among 107 glycerol consuming bacterial isolates from soil samples of Atlantic forest ecosystem, including 6 bacterial isolates with positive result for PHA production after Sudan Black B staining reaction in solid MSM. This isolated bacterial strain was previously referred to as isolate MA13, whose partial 16S rRNA gene sequence (1334 bp) has been deposited in the GenBank database under the accession number KC111959. The alignment results from BLASTn function showed maximum scores for *B. glumae* strains with identity levels of 99%. Therefore, the isolate MA13 has been referred to as *B. glumae* MA13. High similarity levels to 16S rRNA gene sequences of other *Burkholderia* species were detected from the alignment with available sequences of *B. gladioli* (99%), *B. plantarii* (99%), *B. thailandensis* (98%), *B. pseudomallei* (98%), *B. mallei* (98%), *B. vietnamiensis* (97%) and *B. mimosarum* (96%). A phylogenetic tree was constructed using the 16S rRNA sequence of *B. glumae* MA13 and other bacterial species from related taxa (Fig. 1). The molecular evolutionary analysis also showed high similarity to *B. glumae* with a bootstrap confidence value of 100%. Biochemical and morphological characterization of *B. glumae* are given in Table 1, which shows the main results for carbohydrate assimilation tests.

3.2. PHA production from biodiesel byproduct

The 6 isolated bacteria with positive PHA production reaction after Sudan Black B staining were cultivated in MSM containing pure glycerol as sole carbon source and their PHA production capabilities were confirmed after propanolysis reaction and consecutive polymer determination, which showed a PHA constituted of P(3HB) homopolymer from glycerol as sole carbon source. Among these 6 bacterial isolates, the strain MA13 (*B. glumae*) showed the best results of polymer production and intracellular accumulation (Table 2). Therefore, in front of these results *B. glumae* MA13 was selected for further PHA production experiments. Cultivations in MSM containing crude glycerol from biodiesel plant at different concentrations revealed the promising potential of *B. glumae* for PHA production using this carbon source. CDW values ranged from 3.8 to 4.3 g/L with P(3HB) accumulation varying from 31.3 to 41.6% CDW, which resulted in P(3HB) production averages of

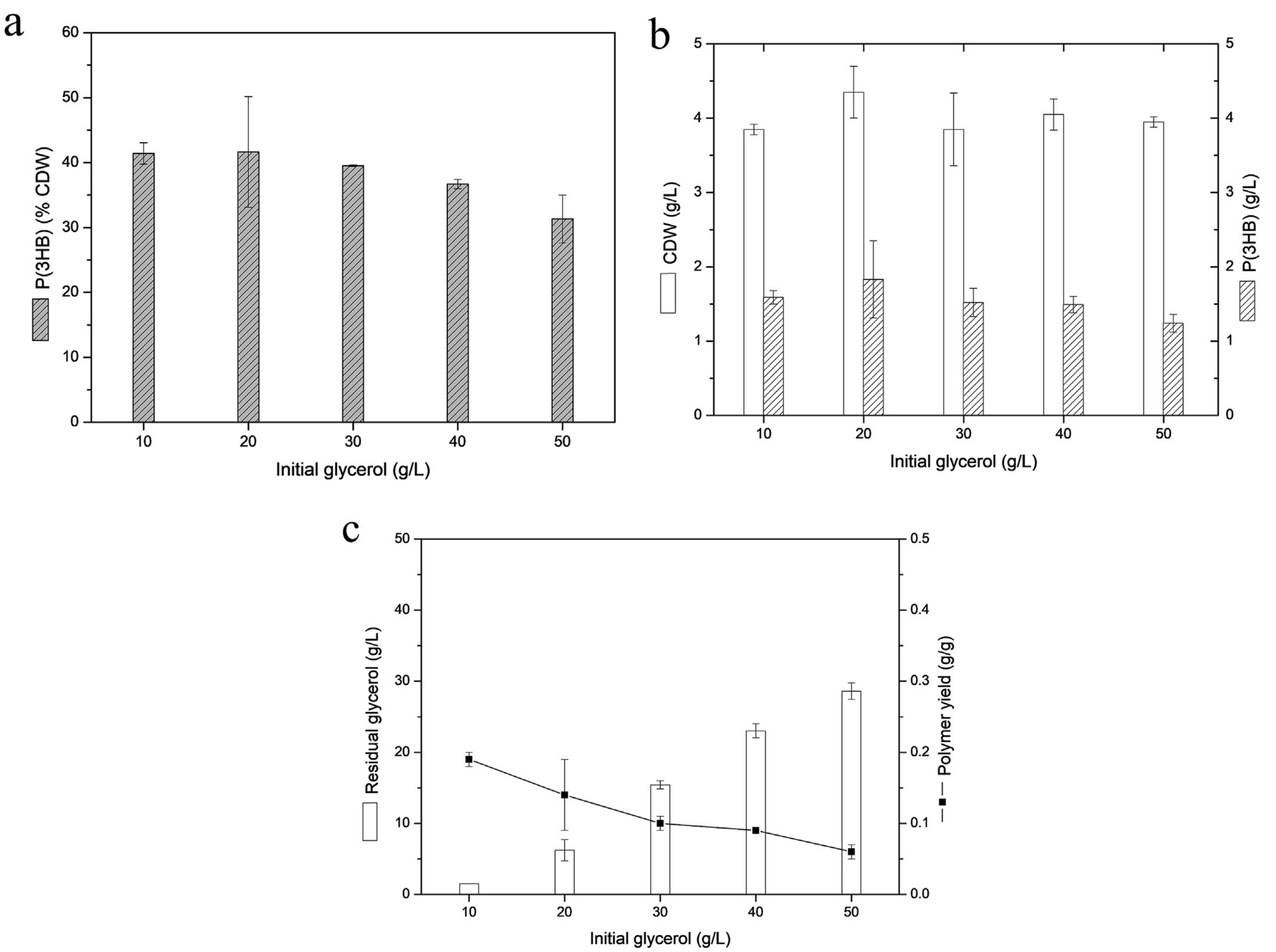

Fig. 2. Shake flask experiments for P(3HB) production from MSM containing different crude glycerol concentrations after 72 h at 30 °C. Profile of (a) intracellular P(3HB) accumulation; (b) CDW and P(3HB) production; and (c) Residual glycerol and Polymer yield.

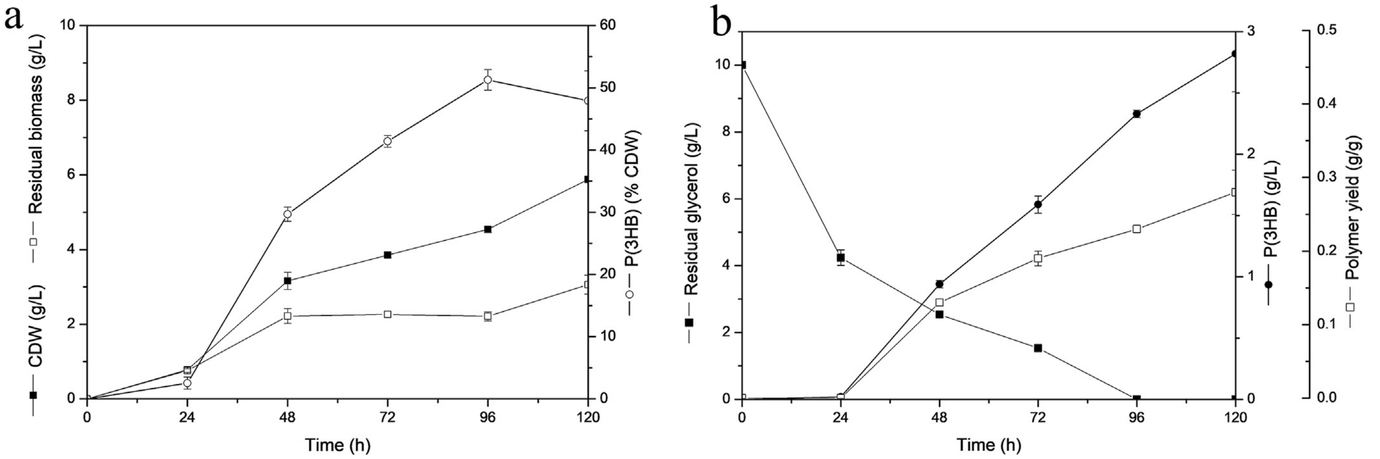

Fig. 3. Shake flask experiments for kinetic of P(3HB) production from MSM containing crude glycerol during 120 h at 30 °C. Profile of (a) CDW, Residual biomass and intracellular P(3HB) accumulation; (b) Residual glycerol, P(3HB) production and Polymer yield.

1.2–1.8 g/L (Fig. 2). Best polymer yield was obtained using 10 g/L crude glycerol with a value of 0.19 P(3HB) grams per grams of consumed glycerol. P(3HB) production values from crude glycerol were similar to those obtained from pure glycerol. These results demonstrate that *B. glumae* MA13 is an adapted bacterial strain to synthesize PHAs from crude glycerol as sole carbon source and free of pretreatment.

Kinetic experiments in MSM containing 10 g/L crude glycerol showed a continuous increase of P(3HB) production up to 120 h, which was prominent after 24 h cultivation (Fig. 3). Maximum P(3HB) production of 2.8 g/L was obtained after 120 h cultivation, whereas

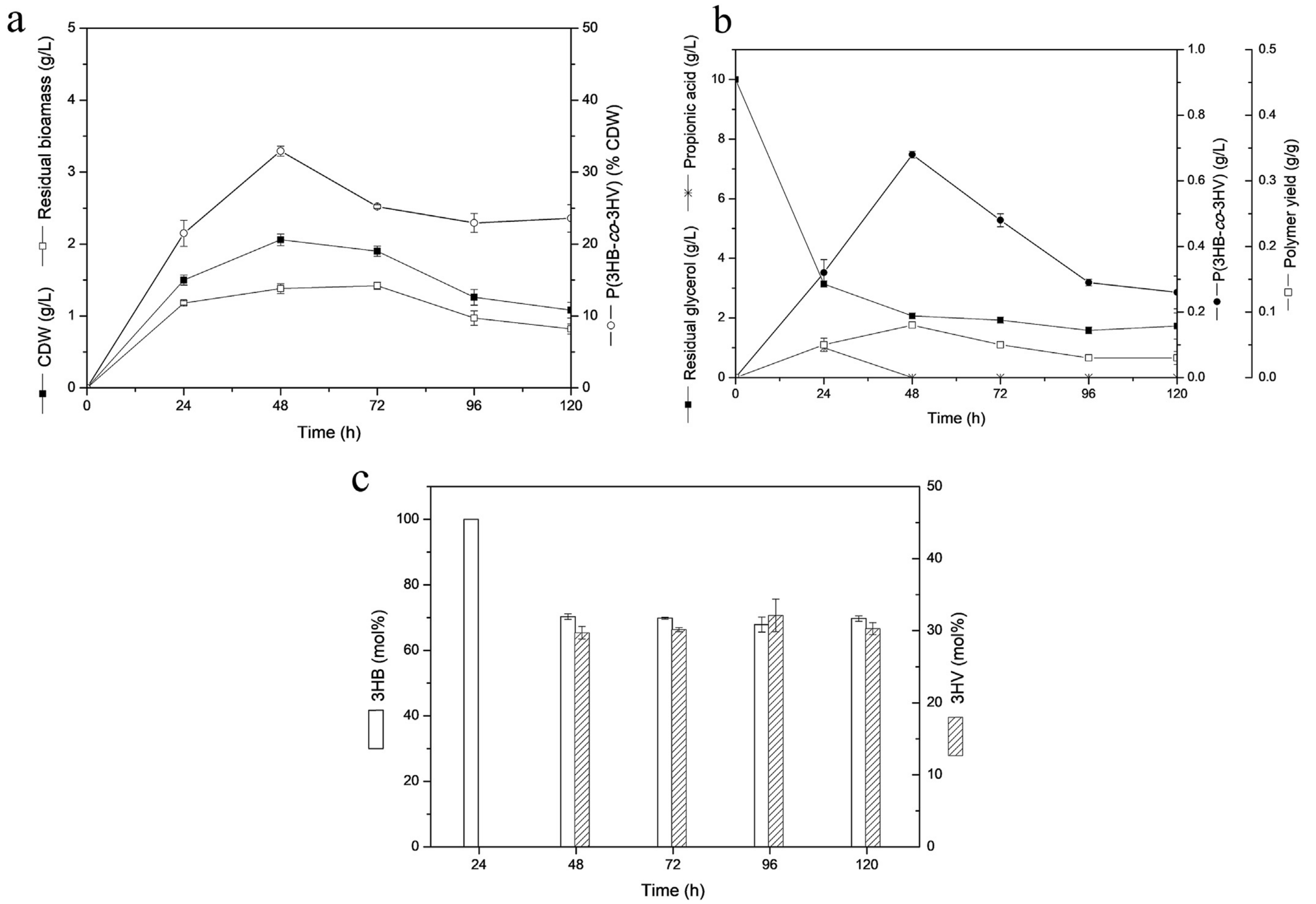

Fig. 4. Shake flask experiments for kinetic of P(3HB-*co*-3HV) production from MSM containing crude glycerol added propionic acid during 120 h at 30 °C. Profile of (a) CDW, Residual biomass and intracellular P(3HB-*co*-3HV) accumulation; (b) Residual glycerol and propionic acid, P(3HB-*co*-3HV) production and Polymer yield; (c) 3HB and 3HV molar percentage content.

intracellular polymer accumulation reached a maximum of 51.3% CDW after 96 h, when carbon source was exhausted. Likewise, P(3HB) yield was highest after 120 h (0.28 g/g), evidencing that periods of cultivation aiming at the best results for PHA production from crude glycerol by *B. glumae* MA13 under the tested cultivation parameters should range between 96 and 120 h. If it is considered the maximum theoretical yield of 0.47 g/g for P(3HB) production from glycerol via Acetyl-CoA pathway (Moralejo-Gárate et al., 2011), the polymer yield obtained from P(3HB) kinetic experiments utilizing crude glycerol achieved 59.6% of maximum theoretical yield. Although crude glycerol was a promising carbon source as pure glycerol for P(3HB) synthesis by *B. glumae* MA13, the increasing concentrations of this biodiesel by-product from 20 to 50 g/L impaired the polymer production values. Cavalheiro et al. (2009) have also reported the negative effect of increasing crude glycerol concentrations on PHA production by *Cupriavidus necator*, which they ascribed to the presence of salts impurities. The same was observed by Mothes et al. (2007) for *C. necator* and *Paracoccus denitrificans* utilizing crude glycerol for PHA production, with a negative effect on the bacterial growth, which resulted in lower polymer yields. However, the decreasing values of PHA production by *B. glumae* MA13 were directly related to the negative effect of increasing crude glycerol concentrations on intracellular P(3HB) accumulation, since similar CDW values were obtained for all tested carbon source concentrations.

On the other hand, the metabolic role of NaCl stress has been investigated on 3-ketothiolase activity (Natarajan et al., 1995), which is involved into the condensation of 2 molecules of Acetyl-CoA as a step for P(3HB) synthesis (de Paula et al., 2018). The 3-ketothiolase activity increased to 350% under salts stress compared to control cultivation of *Rhizobium* (Natarajan et al., 1995). Even though the P(3HB) production by *B. glumae* was impaired by increasing crude glycerol concentrations, similar values of polymer production and intracellular accumulation to pure glycerol cultivations were obtained from crude glycerol concentrations lower than 30 g/L, and the best results of P(3HB) production were observed from crude glycerol after 96 h cultivation. Although sodium methoxide and sodium hydroxide are preferred catalysts for biodiesel production via transesterification reaction of fats and oils (Knothe and Razon, 2017) and the increasing of crude glycerol concentrations affected exclusively the intracellular PHA accumulation by *B. glumae*, further experiments are necessary to elucidate the role of sodium ions on PHA synthesis by this new isolated strain, since there are no reports about the salts stress on key enzymes involved in the PHA synthesis by *B. glumae*.

Cultivations performed in MSM containing crude glycerol added propionic and hexanoic acids resulted in the copolymers P(3HB-*co*-3HV) and P(3HB-*co*-3HHX), respectively. Since propionic acid is also an antimicrobial agent commonly used for food conservation (Steinbüchel and Lütke-Eversloh, 2003), the CDW values observed from crude glycerol plus propionic acid were lower than remaining experiments performed in this study with a maximum of 2 g/L (Fig. 4). Intracellular polymer accumulation was also affected by propionic acid addition to the media with values ranging from 21.5 to 32.9% CDW, which resulted in lower P(3HB-*co*-3HV) production with a maximum of 0.7 g/L. However, the copolymer synthesized by *B. glumae* comprised a 3HV

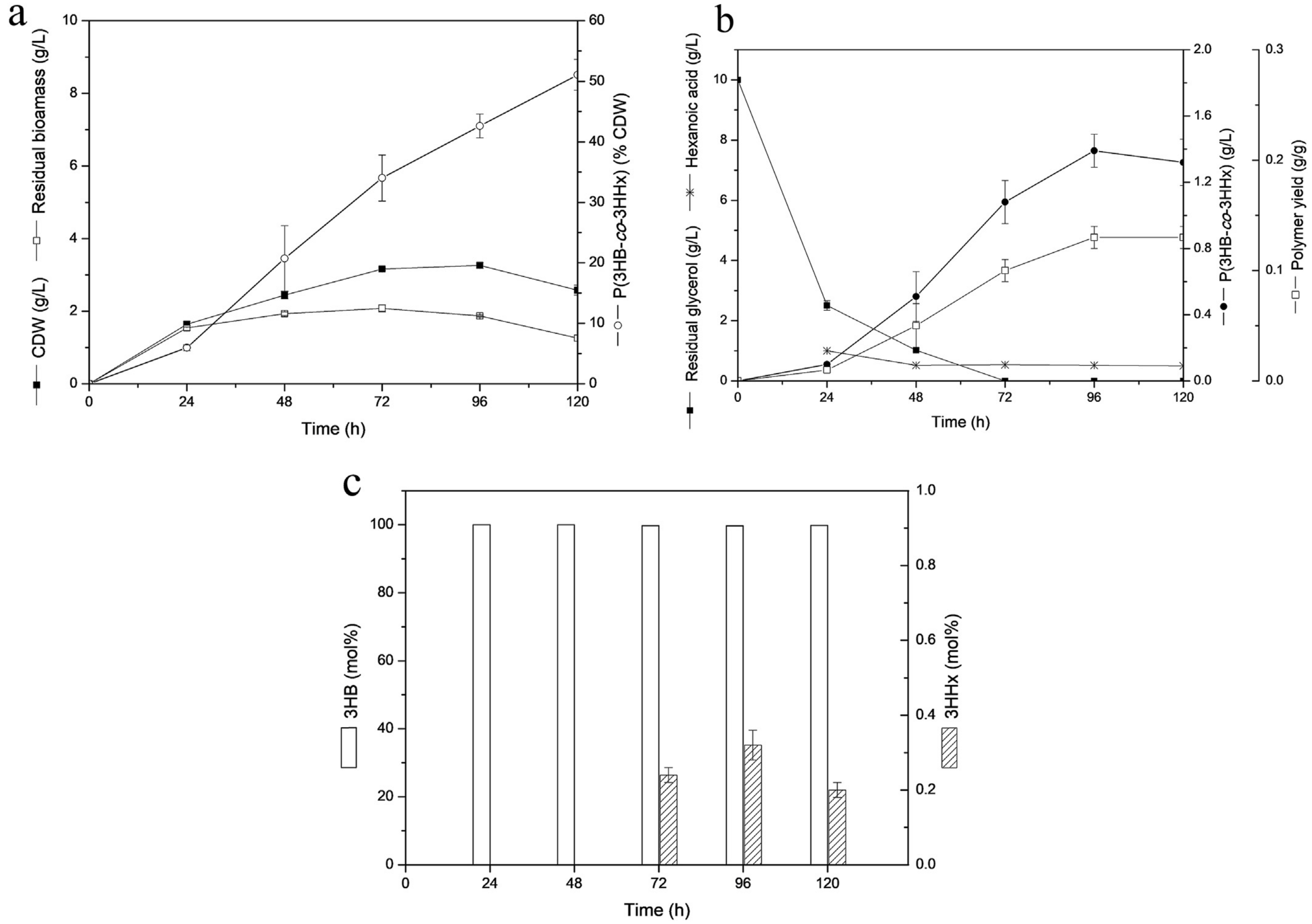

Fig. 5. Shake flask experiments for kinetic of P(3HB-*co*-3HHx) production from MSM containing crude glycerol added hexanoic acid during 120 h at 30 °C. Profile of (a) CDW, Residual biomass and intracellular P(3HB-*co*-3HHx) accumulation; (b) Residual glycerol and hexanoic acid, P(3HB-*co*-3HHx) production and Polymer yield; (c) 3HB and 3HHx molar percentage content.

Table 3
PHA production from different carbon sources by *Burkholderia glumae* MA13. Mean values from shake flask experiments after 72 h.

Carbon source	CDW (g/L)	PHA (%CDW)	PHA (g/L)	Polymer yield[a] (g/g)	Residual biomass[b] (g/L)
Sugar cane molasses	3.21	51.41	1.65	0.17	1.56
Waste cooking oil	5.61	49.02	2.75	0.30	2.86
Pure glycerol	4.20	46.27	1.94	0.21	2.26
Crude glycerol	3.85	41.40	1.59	0.19	2.26
Crude glycerol + propionic acid	1.90	25.20 (30.15 mol%[c])	0.48	0.04 (0.16[d])	1.42
Crude glycerol + hexanoic acid	3.16	34.02 (0.24 mol%[e])	1.08	0.11 (0.01[f])	2.08

[a] grams of polymer per grams of carbon source consumption.
[b] cell dry weight deducted of PHA polymer.
[c] molar percentage of 3-hydroxyvalerate monomer.
[d] polymer yield of 3-hydroxyvalerate production per propionic acid consumption.
[e] molar percentage of 3-hydroxyhexanoate monomer.
[f] polymer yield of 3-hydroxyhexanoate production per hexanoic acid consumption.

content varying from 29.7 to 32.1 mol%, which is an interesting result since copolymers containing more than 20 mol% of 3HV exhibit elongation properties suitable for industrial applications (Kim and Lenz, 2001). Hexanoic acid addition to MSM containing crude glycerol resulted in low fractions of 3HHx monomer with a P(3HB-*co*-3HHx) copolymer consisted of 0.2–0.3 mol% 3HHx (Fig. 5). Polymer accumulation achieved a maximum of 51% CDW after 120 h. However, the addition of hexanoic acid to the culture media also negatively affected the bacterial growth with lower CDW values (1.6–3.3 g/L) than those obtained from MSM cultivations using crude glycerol as sole carbon source, which resulted in a maximum P(3HB-*co*-3HHx) production of 1.4 g/L.

In order to verify the ability of *B. glumae* MA13 to synthesize PHAs from other biofuel related byproducts, this bacterial strain was cultivated in MSM containing waste cooking oil or sugarcane molasses as sole carbon sources. Intracellular P(3HB) accumulation values over 50% CDW have been reported for *C. necator* from soybean oil (Pradella et al., 2012) and *Burkholderia sacchari* IPT 189 from sucrose (Rocha et al., 2008). On the other hand, some authors have reported PHA production from different substrates or biofuel related byproducts by an

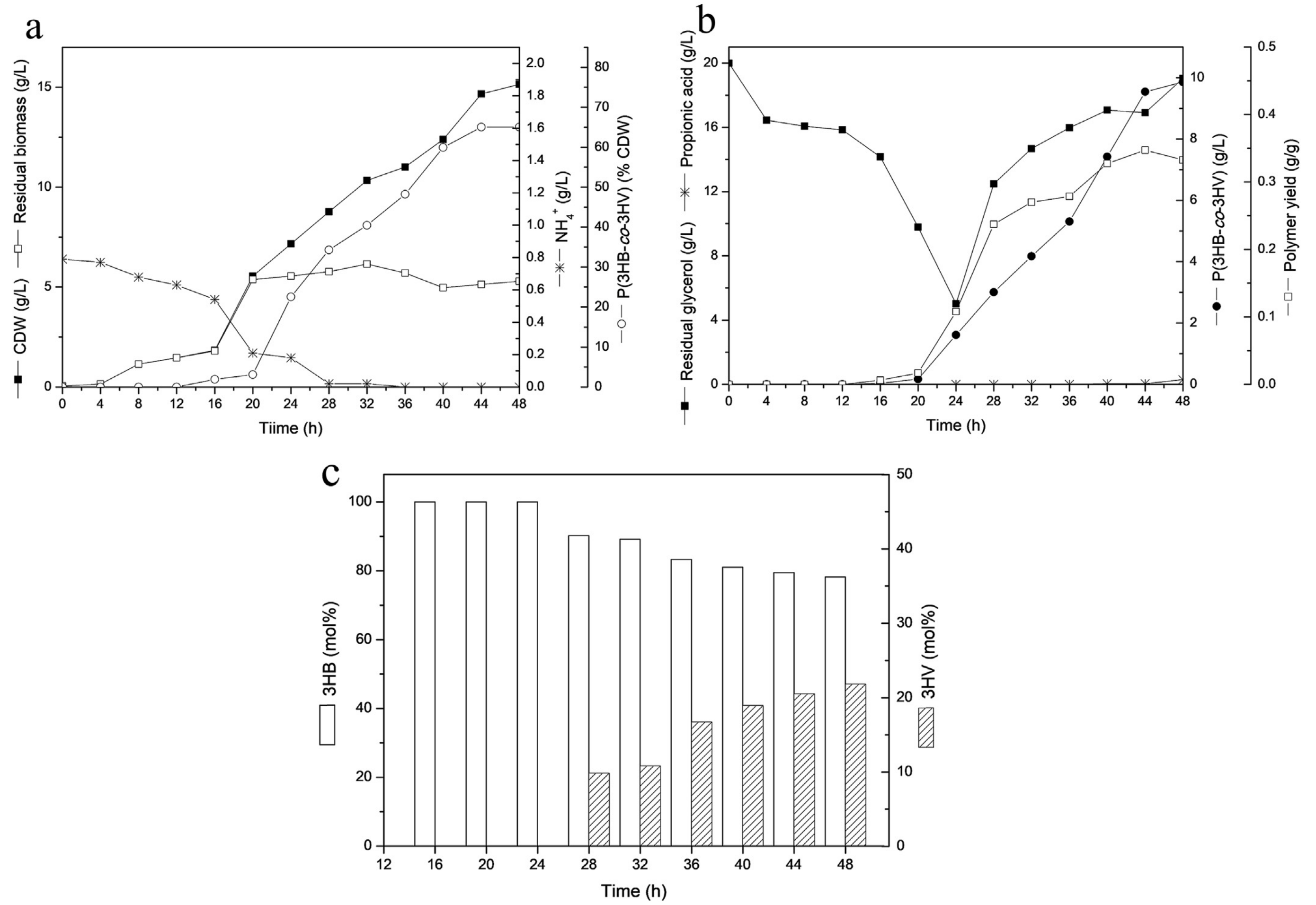

Fig. 6. Fed-batch bioreactor cultivation of *B. glumae* MA13 for P(3HB-*co*-3HV) production from crude glycerol and propionic acid during 48 h at 30 °C. Profile of (a) CDW, Residual biomass, NH_4^+ and intracellular P(3HB-*co*-3HV) accumulation; (b) Residual glycerol and propionic acid, P(3HB-*co*-3HV) production and Polymer yield; (c) 3HB and 3HV molar percentage content.

unique adapted microbial strain. *Pseudomonas* species are well-known for their ability to synthesize mcl-PHAs from a variety of carbon sources, specially oil substrates. Costa et al. (2009) tested different carbon sources for mcl-PHA production by *P. aeruginosa* strains and observed an intracellular polymer accumulation range of 43–50% CDW from waste cooking oil. However, the same authors reported PHA accumulation varying from 5 to 22% CDW from glycerol. Similar results were obtained in the experiments of Phukon et al. (2014) that have reported an intracellular PHA accumulation value of 22.5% CDW by *P. aeruginosa* JQ866912 from crude glycerol as sole carbon source. Additionally, these authors have also observed PHA accumulation values of 14.5% and 13.2% CDW from pure glycerol and sugarcane molasses, respectively. Sen et al. (2019) have reported a maximum intracellular P(3HB) accumulation of 27% CDW from hydrothermal acid pretreated sugarcane molasses by *C. necator*. Table 3 shows the PHA production by *B. glumae* MA13 from different biofuel related byproducts after 72 h cultivation, including crude glycerol, waste cooking oil and sugarcane molasses as sole carbon sources and free of pretreatment, with polymer accumulation values ranging from 41.4 to 51.4% CDW, confirming the ability of this new isolated bacterial strain to produce PHAs from multiple feedstocks.

3.3. Fed-batch bioreactor cultivation

Due to the P(3HB-*co*-3HV) production yields obtained from crude glycerol plus propionic acid by *B. glumae* MA13 in previous shake flask cultivations, a bioreactor experimental set was performed in order to evaluate the scaling-up of polymer production following two cultivation steps, with the latter under feed regimen. First cultivation step started with a 10% inoculum (v/v), which resulted in a short lag phase of 4 h. However, the exponential growth phase was observed from 4 to 20 h cultivation with a maximum specific growth rate of 0.23 h^{-1}, which can be considered a long period compared to the lag phase and possibly it can be attributed to a required adaptation period to crude glycerol as sole carbon source. PHA accumulation started after 12 h cultivation though the polymer production was prominent after 20 h, when values of NH_4^+ concentration were lower than 0.21 g/L and thus confirming the not growth-associated PHA biosynthesis. Pradella et al. (2010) reported higher P(3HB) accumulation values from sucrose by *B. sacchari* IPT 189 under excess carbon and nitrogen limitation. These authors reported a NH_4^+ value lower than 0.19 g/L to trigger the P(3HB) biosynthesis, which is very similar to the limiting NH_4^+ concentration value obtained in this study for the onset of accumulation phase of *B. glumae* MA13. The feed regimen started after 24 h cultivation, when intracellular polymer accumulation was 22.6% CDW and consisted of P(3HB) homopolymer, with a residual biomass value of 5.5 g/L. The crude glycerol and propionic acid solution was constantly fed to the culture broth resulting in a specific feed rate of 0.23 g/(g h). The feed regimen of carbon sources was adjusted to a feed rate sufficient to maintain the glycerol concentration at a range of 10–20 g/L and maximum propionic acid concentration of 1 g/L. P(3HB-*co*-3HV) production was maximum after 48 h cultivation with 9.9 g/L comprising 65.1% CDW (Fig. 6). Hermann-Krauss et al. (2013) reported P(3HB-*co*-3HV) production from pure glycerol by an archaeal strain *Haloferax*

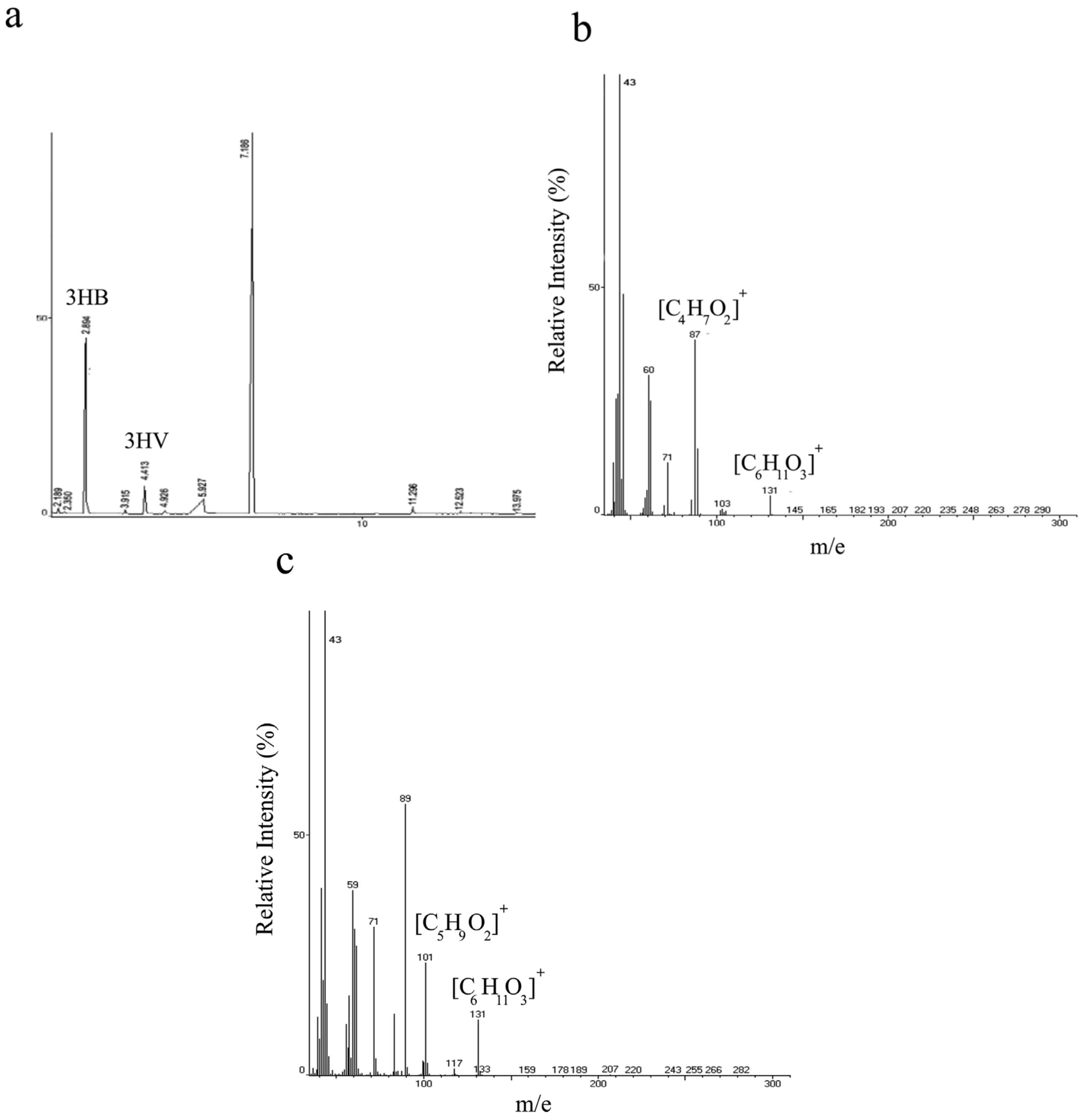

Fig. 7. Gas chromatography coupled with mass spectrometry analysis of extracted P(3HB-*co*-3HV) produced by *B. glumae* MA13.(a) Peaks at retention time 2.95 and 4.46 min were identified as 3HB and 3HV, respectively. Mass spectra of electron ionized propyl-esters of 3-hydroxyalkanoates: (b) 3HB and (c) 3HV.

mediterranei with a copolymer comprising 75% CDW and 10 mol% of 3HV. Lefebvre et al. (1997) have emphasized the importance of oxygen limitation as an additional strategy to obtain higher 3HV yields by *C. necator* using glucose and propionic acid as carbon sources, resulting in a copolymer consisted of 25 mol% of 3HV. However, *B. glumae* MA13 has achieved a 3HV molar percentage of 21.8% from crude glycerol and propionic acid under a dissolved oxygen of 20% saturated air.

As aforementioned, P(3HB-*co*-3HV) copolymers constituted of more than 20 mol% 3HV display physicochemical properties that make then more suitable for industrial applications. Furthermore, *B. glumae* MA13 synthesized a copolymer with high 3HV conversion yields achieving 1.04 g of 3HV monomer per gram of consumed propionic acid, which is 76.7% of the maximum theoretical yield of 1.35 g/g (Gomez et al., 1996). Therefore, *B. glumae* not only produces P(3HB-*co*-3HV) copolymer from crude glycerol and propionic acid with suitable 3HV content, but also it is able to synthesize this monomer exhibiting high conversion yields and so sparring the expenses with this propionigenic precursor as an additional economic strategy for P(3HB-*co*-3HV) synthesis besides the crude glycerol utilization. A variety of bacteria have achieved poor 3HV yields not exceeding 0.10 g/g. The predominance of oxidative pathways to generate energy and the conversion of propionic acid into 3HB monomers instead 3HV are the main metabolical shifts that lead to low conversion yields of propionic acid into 3HV monomers (Gomez et al., 1996; Silva et al., 2000). The 3HV conversion yields obtained by *B. glumae* MA13 wild strain can be compared to those obtained by mutants strains of *B. sacchari* (Rocha et al., 2008) and *Pandoraea* sp. (de Paula et al., 2017) which reached 81 and 86% of maximum theoretical yield, respectively.

The bioreactor experiment resulted in a maximum volumetric productivity of 0.22 g/(L h) after 44 h cultivation, when it was observed a P(3HB-*co*-3HV) production of 9.5 g/L, which was 65% of total CDW of 14.7 g/L. Cavalheiro et al. (2012) have obtained similar volumetric productivity of 0.25 g/(L h) of P(3HB-*co*-4HB-*co*-3HV) terpolymer with an intracellular polymer accumulation of 36.9% CDW from crude glycerol, γ-butyrolactone and propionic acid by a glucose consuming mutant of *C. necator* (*C. necator* DSM 545), while Kachrimanidou et al. (2014) have reported the production of P(3HB-*co*-3HV) copolymer from crude glycerol, sunflower meal hydrolysates and levulinic acid by *C. necator* DSM 7237 with a volumetric productivity of 0.25 g/(L h). P (3HB-*co*-3HV) produced by *B. glumae* MA13 from crude glycerol and propionic acid in bioreactor experiment was extracted from lyophilized cell pellets by chloroform reaction and analyzed by GC-MS in order to confirm the monomer composition of synthesized polymer. Fig. 7 shows the chromatogram and mass spectra of copolymer produced by *B. glumae* MA13. The peaks at 2.95 and 4.46 min were identified as 3HB and 3HV, respectively, according to the mass spectra of propyl-esters of 3-hydroxyalkanoates. The fragment m/e 131 is characteristic of propyl-esters formed by α-cleavage of the hydroxyl functional group (Lee and Choi, 1995), whereas the monomers 3HB and 3HV were identified by analysis of fragments of m/e [M-59] (Silva-Queiroz et al., 2009), resulting in the fragment m/e 87 for 3HB and the fragment m/e 101 related to 3HV as mass difference values between the total molecule and the assigned ion.

4. Conclusions

The results obtained in this study revealed *B. glumae* MA13 as a new and promising PHA-producing bacterial strain, which is able to convert not only crude glycerol but also waste cooking oil and sugarcane molasses into PHA biopolymers showing high polymer yields. All these evaluated carbon sources are low value substrates and their utilization for biopolymer production is an integrated solution for bio-based industries, which have focused their efforts in an associated production set of biofuels and biopolymers as a renewable and ecologically friendly alternative to petrochemical-based fuels and plastics. Due to the promising results obtained in this study by the new isolated *B. glumae* MA13, the authors aim to perform future experiments in order to optimize cultivation parameters, test additional carbon sources and analyze the thermo-mechanical properties of the synthesized polymer besides to analyze the economic viability to implement a copolymer produced by *B. glumae* MA13 from industrial byproducts.

Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors thank São Paulo State Research Foundation (FAPESP) and Coordination for the Improvement of Higher Education Personnel (CAPES) for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101268.

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