AsPac J. Mol. Biol. Biotechnol. 2019
Vol. 27 (3) : 64-72

Engineering of *Bacillus megaterium* for improving PHA production from glycerol

Javier Ricardo Gómez Cardozo[a], Rodrigo Velasco Bucheli[b], Carlos del Cerro Sánchez[c], Isabel de la Mata Riesco[d], Amanda Lucía Mora Martínez[e*]

[a]*Facultad de Ciencias, Universidad Nacional de Colombia, Medellin, Colombia*
[b]*Enzyme Development, Roche Diagnostics GmbH, Penzberg, Germany*
[c]*Microbial and Plant Biotechnology Department, Centro de Investigaciones Biológicas CIB-CSIC, Madrid, Spain*
[d]*Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense de Madrid, Madrid, Spain*
[e]*Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Medellin, Colombia*

Received 9th May 2019 / Accepted 24th June 2019

Abstract. There are a few PHA-producer bacteria that can uptake glycerol to produce this biopolymer. Among them, *Bacillus megaterium* LVN01 has demonstrated to be able to grow up using glycerol as a carbon source. Glycerol dehydrogenase (GD) plays a key role in the synthesis of PHA from glycerol. In this study, the improvement of glycerol uptake by a recombinant strain of *B. megaterium* carrying pHT01-*bmgd* was evaluated in order to enhance PHA production. The biomass and PHA production were evaluated and compared to wild-type. It was determined that the PHA produced by both strains was PHB and the highest improvement in PHB yield was 226% at 30 h.

Keywords: *Bacillus megaterium*, glycerol, glycerol dehydrogenase, polyhydroxyalkanoates

INTRODUCTION

During the last decades, biofuels have been considered as important alternatives to stop global warming, however, this endeavor contrasts with waste glycerol production, which has emerged as an important contaminant byproduct (Kalia *et al.*, 2016; Mohapatra *et al.*, 2017).

A few wild-type bacteria are able to synthesize PHA from glycerol as the sole carbon and energy sources, such as *Bacillus* (Okwuobi and Ogunjobi, 2013), *Burkholderia* (Rodríguez-Contreras *et al.*, 2015), *Cupriavidus* (Campos *et al.,* 2014), *Halomona*s (Kawata and Aiba, 2010), *Novosphingobium* (Teeka *et al.,* 2012), *Pseudomona*s (Pappalardo *et al.,* 2014; Ashby *et al.,* 2015) and *Zobellella* (Ibrahim and Steinbüchel, 2010).

Polyhydroxyalkanoates are macromolecules synthesized by many bacteria, and it is accumulated as intracellular granules to levels as high as 90% of cell dry weight (Reddy *et al.,* 2003). It is stored in those microorganisms under conditions of nutrient stress in the presence of an excess carbon source (Reddy *et al.,* 2009; Bhuwal *et al.,* 2014). It has been demonstrated that *Bacillus megaterium* is able to produce different kinds of PHA from many feedstocks (including glycerol), such as poly(3HB-*co*-3HV) and PHB (Moreno *et al.,* 2015; Porras *et al.,* 2017).

Glycerol is converted into PHA by an oxidative pathway via the NAD^+-dependent enzyme glycerol dehydrogenase that catalyzes the

*Author for correspondence: Amanda Lucía Mora Martínez, Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Medellin, Colombia. Email – almora@unal.edu.co

conversion of glycerol to dihydroxyacetone. The glycolytic enzyme dihydroxyacetone kinase phosphorylates this ketone, which is then incorporated into the glycolysis pathway, and eventually transformed to pyruvate (da Silva *et al.,* 2009; Zhu *et al.,* 2013). The pyruvate dehydrogenase transforms pyruvate to acetyl-CoA. Then two molecules of acetyl-CoA are condensed by β-ketothiolase (PhaA) into acetoacetyl-CoA. The acetoacetyl-CoA reductase (PhaB) reduces acetoacetyl-CoA to (*R*)-3-hydroxyalkyl-CoA. Finally, PHA synthase (PhaC) polymerize 3-hydroxyalkyl-CoA to PHA (Figure S1).

Bacillus megaterium is a well-known PHA-producer bacterium able to convert different carbon sources into short-chain-length (scl)-polymers (e.g. poly(3-hydroxybutyrate)) or scl-copolymers (e.g. poly(hydroxybutyrate-*co*-hydroxyvalerate)) (Możejko-Ciesielska and Kiewisz, 2016). The classical approach to improve the PHA production by conventional fermentation strategies is not good enough in order to achieve yields suitable for industrial purposes. For instance, modifications of the C/N ratio or dissolved oxygen could induce forming endospore, which reduces the yield of PHA production (Koller *et al.,* 2016).

Nowadays, metabolic engineering is one of the most powerful tools among the strategies implemented to increase yield in biotechnological processes, and in this case, it is implemented either to enhance the use of the substrates (Povolo *et al.,* 2010) or to avoid the degradation of the PHA, once it is synthesized (Povolo *et al.,* 2015).

The advantages of using a PHA producer such as *B. megaterium* are an absence of toxic lipopolysaccharides (biologically safe) (Stewart *et al.,* 2006), and high PHA yield and broad spectra of synthesized biopolymers from many sources (Kumar *et al.,* 2013). Likewise, metabolic manipulation in *B. megaterium* has been reported, such as engineered recombinant strains with easy PHA recovery by self-disruptive mechanism (Hori *et al.,* 2002), tailored PHA producers (Singh *et al.,* 2015), or obtaining functional intracellular materials (Grage *et al.,* 2017), among others.

In this study, we report the obtaining of a recombinant clone *B. megaterium Bm*GD capable to accumulate PHA and grow up faster than the wild-type, using glycerol as the carbon source. This recombinant strain is suitable for industrial application since it is able to use a cheap and contaminant carbon source, while it displays an improved PHA-production in a short period of time.

MATERIALS AND METHOD

Bacterial strains, plasmids and growth conditions. *Bacillus megaterium* LVN01, Colección Española de Cultivos Tipo (CECT) 9345 (Sánchez *et al.,* 2012), *Escherichia coli* DH5α (F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1), and the plasmid pHT01 (Amp^r, Cm^r, *ori* ColE1) were used in this study. *Bacillus megaterium* LVN01 was used as the bacterial host and for expression. *Escherichia coli* DH5α was used as the host for plasmids and constructions. The vector pHT01 (MoBiTec GmbH) was used in all transformation experiments. *Bacillus megaterium* LVN01 and *E. coli* DH5α cells were grown in LB medium consisting of 1% bacto-tryptone 0.5% yeast extract and 1% NaCl, and incubated at 30°C and 37°C, respectively. Solid media contained 1.75% (w/v) agar. Transformants of *B. megaterium* obtained by electroporation, and from *E. coli* obtained by heat-shock were resuspended in LB medium. Antibiotics were supplemented as required at the following concentration: for *B. megaterium Bm*GD chloramphenicol 5 μg mL^{-1} and for recombinant clones of *E. coli* ampicillin 100 μg mL^{-1}.

Construction of recombinant B. megaterium LVN01 strains. Plasmids were isolated using the PureLink® Quick Plasmid Miniprep Kit (Life Technologies). The DNA was analyzed by electrophoresis in 0.8% (w/v) agarose gel slices mixing with GelRed Nucleic Acid Gel Stain (Biotium) and GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific) used as the marker. All DNA fragments were isolated from agarose gel using the GFX PCR DNA and Gel Band Purification Kit (GE healthcare). Restriction enzymes (*Bam*HI and *Sma*I) and T4 DNA ligase (New England BioLabs) were used according to instructions provided by the supplier. The *bmgd*

gene (1.13 kbp) PCR product was amplified from the chromosomal DNA of *B. megaterium* LVN01 (GenBank: QJGY00000000.1). The PCR product was subsequently purified and digested by restriction enzymes. Thereafter, *bmgd* was cloned in pHT01 (7.96 kb). The ligation product was transformed in *E. coli* DH5α for amplification. The mutant plasmid was isolated from *E. coli* transformants, and it was finally transformed in *B. megaterium* LVN01 by electroporation.

The PCR amplification of *B. megaterium* glycerol dehydrogenase (*bmgd*) gene was performed using a PCR program in the MJ Mini Gradient Thermal Cycler (BioRad). Denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 53.6ºC for 1 min and elongation at 72ºC for 2.5 min. A final elongation at 72ºC for 15 minutes was carried out.

The primers used were *Bm*GD1 5'-TCCCCCGGGGGATTATATTAACCTCTTC-3', *Bm*GD2 5'CGGGATCCCGATGAGGAAAG CATTTATTAG-3', *Bm*GD-Nt 5'-CATCGAAA GCACCCTCTGGT-3', and *Bm*GD-Ct 5'-GTGG ATTAGCTGGCGCACAT-3'.

PHB production. For PHB analysis, *Bacillus megaterium* strains were inoculated in Minimal Salt Medium (MSM), which contains 20.0 g L^{-1} glycerol, 1.0 g L^{-1} yeast extract, 1.5 g L^{-1} KH_2PO_4, 3.6 g L^{-1} Na_2HPO_4, 0.8 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 1.0 mL of trace element solution. The trace element solution contained: 10.0 g L^{-1} $FeSO_4 \cdot 7H_2O$, 2.25 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 1.0 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 g L^{-1} $MgSO_4 \cdot 5H_2O$, 2.0 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.23 g L^{-1} $Na_2B_4O_7 \cdot 10H_2O$, 0.1 g L^{-1} $(NH_4)_6Mo_7O_{24}$ and 10.0 mL 35.0% HCl (Kim *et al.,* 1994). Chloramphenicol (5.0 μg mL^{-1}) was supplemented when necessary. When the OD_{600} of the culture broth was equal to 1.0, the expression of *Bm*GD in the bacterium was induced with 1.0 mM IPTG and further incubated at 30°C. That moment was considered as $t_0 = 0$ h, and at different time were collected samples for analysis.

Analytical procedures.

Determination of biomass and PHA extraction.

Samples of 50 mL of culture broth were taken and centrifuged in pre-weighed plastic screw cap tube for 15 min at 8000 *xg* at 4°C. Pellets were frozen and lyophilized and gravimetric difference against empty tube was calculated. PHA extraction was carried out in centrifuged cells by the method of Jacquel *et al.* (Jacquel *et al.,* 2008) with some modifications. For each 20 mg of biomass 1 mL of chloroform and 1 mL of hypochlorite (5% v/v) were used, the mixture was incubated at 35°C for 2 h. The mixture was centrifuged (10000 *xg*, for 10 min at 20°C), the supernatant was discarded, and the dissolved polymer in chloroform was recovered by solvent evaporation.

PHA Quantification.

The PHA quantification was carried out by crotonic acid method with some modifications. For each 0.1 mg of PHA recovered from biomass, 1 mL of 98% sulfuric acid was added and heated at 100°C for 10 min. The absorbance was measured at 235nm in a UV-VIS spectrophotometer. Poly(3-hydroxybutyric acid) (Sigma-Aldrich) was used for the standard curve. The PHA yield was calculated using a calibration curve (Fig. S2), and the improvement in the production was calculated as follows:

$$\text{PHA yield (mg } L^{-1}) = (A_{235} - 0.0604)/0.1885 \quad \textbf{(1)}$$

$$\text{Improvement (\%)} = [\text{PHA in } Bm\text{GD (mg } L^{-1}) / \text{PHA in LVN01 (mg } L^{-1})] * 100\% \quad \textbf{(2)}$$

Fourier Transform-Infrared Spectroscopy (FT-IR).

The polymer extracted from *B. megaterium* LVN01 and *B. megaterium Bm*GD, as well as the commercial PHB (Sigma-Aldrich), were analyzed by FT-IR. Spectra were recorded using a Spectrum Two FT-IR spectrometer (PerkinElmer). For each measurement, 32 scans were acquired and averaged in the range of 4000 to 400 cm^{-1} with a spectral resolution of 4 cm^{-1}.

Nuclear Magnetic Resonance (NMR).

In order to know the chemical structure of the PHA obtained from *B. megaterium Bm*GD, and compare this compound with the commercial

PHB, an analysis was carried out by one-dimensional nuclear magnetic resonance of proton (^{1}H) and carbon (^{13}C). The spectra were taken on a Bruker 300 NMR spectrometer at 300 MHz, using tetramethylsilane (TMS) as the internal standard. For each measurement, 5 to 10 mg of sample was taken and dissolved in $CDCl_3$.

RESULTS

Bacillus megaterium LVN01 genome. *Bacillus megaterium* LVN01 is a Gram-positive bacterium able to produce PHA. Its draft genome was obtained from a shotgun library constructed and sequenced using Illumina Hiseq 2000 Instruments (Macrogen Inc., Korea). The genome comprises 5.22 Mbp with 37.8% G+C content, obtaining 4169 contigs from 1.5 x 10^7 reads by Newbler 2.5.3. Twenty-one RNA genes (4 rRNA and 17 tRNA) and 4846 coding sequences (CDS) were detected by RAST (Aziz *et al.,* 2008). Regarding CDSs, 1758 were assigned with putative functions and 1715 were classified as hypothetical proteins, including at least 22 enzymes that could play important roles in the biological generation of PHA (data not shown). According to antiSMASH (Medema *et al.,* 2011), this genome contains at least 6 clusters associated with siderophores, terpenes, polyketide synthases (T3PKS) and phosphonates biosynthesis. Despite the average value of cluster in *B. megaterium* is 11, it is important to point out that the estimation is 6, according to the assessment of complete genomes of this strain (Liu *et al.,* 2011; Eppinger *et al.,* 2011; Johnson *et al.,* 2015). Table S1 shows the genomic features of *B. megaterium* LVN01 in comparison to different strains of *B. megaterium* reported in public databases (last update: 29-11-2018).

Furthermore, genome analysis of *B. megaterium* LVN01 using RAST detected 472 subsystems related in the metabolism of carbohydrates, 32 directly related with glycerol metabolism and 149 with fatty acids and lipids, among others (Figure S3).

As was expected, the comparison of its 16S rRNA gene-sequence with other conserved sequences available in NCBI database indicated that this strain is taxonomically close to other *B. megaterium* strains (Figure S4). Similarly, it was possible to detect in *B. megaterium* LVN01 draft genome similar genes that belong to this strain, such as spore coat (*CotB*, *CotD*, *CotE*, *CotF*, *CotH*, *CotJA*, *CotJB*, *CotJC*, *CotP*, *Cot*R, *CotS*, *CotX*, *CoxA*, *GerQ*, *SafA*, *YaaH*, *YmaG*, and *YxeE*), genes involved in gas vesicle biosynthesis *gvp*, operon I (*gvpBRFGLSKJ*) and operon II (*gvpAPQBRNFGLSKJTU*) (Eppinger *et al.,* 2011).

Glycerol dehydrogenase-encoding gene (bmgd) from B. megaterium LVN01. The sequence of *bmgd*-encoding gene was detected and analyzed by BLAST from NCBI through comparison with glycerol dehydrogenases (GD) from other *Bacillus megaterium* (Figure S5). The *bmgd*-encoding gene has a size of 1134 bp, GC-content 37.9%, and it is located within the contig 167 (GenBank: QJGY01000167.1). Further analysis of *B. megaterium* LVN01 glycerol dehydrogenase (*Bm*GD) sequence displays that it has 97% of identity with the GD from *B. megaterium* WSH-002 (Liu *et al.,* 2011) and high identity (99%) with the GD from *B. megaterium* QM B1551 (Eppinger *et al.,* 2011) and *B. megaterium* JX285 (Johnson *et al.,* 2015).

In particular, *Bm*GD has 377 residues, a molecular mass of 40.15 kDa, and a predicted isoelectric point of 4.72. The secondary structure of *Bm*GD predicted by PredictProtein (Yachdav *et al.,* 2014) displays 52.52% of helix, 9.02% of strand and 38.46% of loops.

Regarding its catalytic pocket, it has been reported the crystal structure of *Bacillus stearothermophilus* glycerol dehydrogenase (GlyDH), and it has D39, S127, L129 and Y133 as residues in the binding site for NAD^+, and D123, D173, H256, H274 as amino acids in the glycerol binding site (Ruzheinikov *et al.,* 2001). The protein alignment with *Bm*GD shows that its binding site for NAD^+ could be formed by D40, S123, S125, and Y129, whereas D119, D169, H264, and H281 are part of its glycerol binding site (Figure S6).

The *bmgd* gene was amplified and cloned in pHT01 plasmid between *Sma*I and *Bam*HI restriction sites. This shuttle vector was employed to obtain the recombinant strain *B. megaterium Bm*GD, which overexpress *Bm*GD. The mutant plasmid was corroborated by PCR and agarose gel, and the recombinant strain by colony PCR.

The *Bm*GD expression band was also visualized by SDS-PAGE (Figure S7).

Microbial growth using glycerol as carbon and energy source. The growth and PHA yield of *B. megaterium* LVN01 and *B. megaterium Bm*GD were compared using glycerol as the carbon source. The dry-weight comparison between both strains using glycerol highlights that the recombinant strain is producing more biomass in less time than wild-type (Figure 1). Similarly, the PHA content in the recombinant clone displays a clear improvement of the PHA yield in comparison to the wild-type during the evaluation under the operating conditions (Figure 2). The PHA yield reached the highest improvement (226%) between 24 h and 30 h.

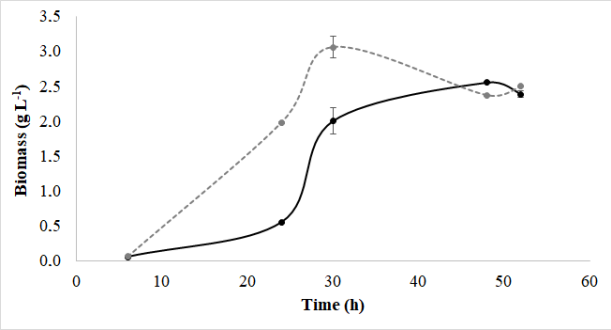

Figure 1. Dry weight cell of *B. megaterium* LVN01 (solid line) and *B. megaterium Bm*GD (dotted line) in waste glycerol (C/N ratio 44.9 mol mol^{-1}; 30ºC, pH 7.0).

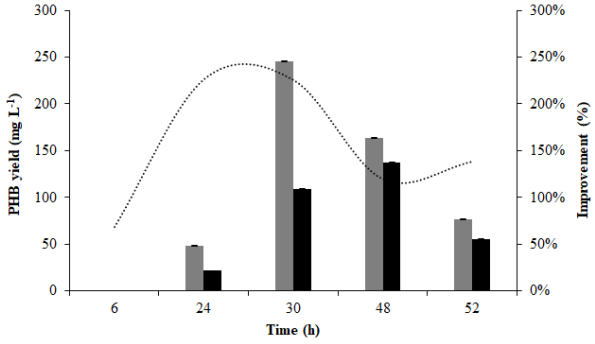

Figure 2. Poly (3-hydroxybutyric acid) yield of *B. megaterium* LVN01 (black) and *B. megaterium Bm*GD (gray) in glycerol (C/N ratio 44.9 mol mol^{-1}; 30ºC, pH 7.0). The dotted line represents the improvement of the process at different times.

A similar approach was carried out regarding the PHA content (Table S2). This analysis highlighted that the recombinant clone is not only able to grow fast (Figure 1) and obtain higher PHA yield (Figure 2), but it also contains a higher amount of PHA into the cells in comparison to the wild-type (Figure 3). This improvement is more evident after 30 h of incubation, and it remains higher thereafter.

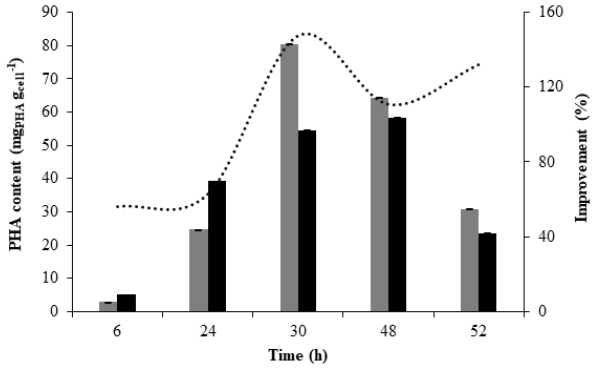

Figure 3. Poly (3-hydroxybutyric acid) content in *B. megaterium* LVN01 (black) and *B. megaterium Bm*GD (gray) in glycerol (C/N ratio 44.9 mol mol^{-1}; 30ºC, pH 7.0). The dotted line represents the improvement in the accumulation at different times.

Fourier transform infrared spectra for PHA from B. megaterium LVN01. Fourier transform infrared (FT-IR) spectra from commercial PHB and PHA produced by both *B. megaterium* strains from glycerol were compared (Figure 4). The absorption peaks at 1720 cm^{-1} and 1280 cm^{-1}, indicate the ester carbonyl group (C=O) and (C-O) ester stretching, respectively. The peaks between 1450 cm^{-1} and 800 cm^{-1} are due to methyl (CH_3) and methylene (CH_2) deformations and ester stretching (C-O). In this case, it is possible to observe some characteristics bands for PHB, such as the bands at 1228 cm^{-1}, 1380 cm^{-1}, and 1180 cm^{-1} are CH_2, CH_3, and C–O–C groups, respectively (Sathiyanarayanan *et al.,* 2013).

Nuclear magnetic resonance (NMR). The analysis of the NMR spectra corresponding to ^{13}C and ^{1}H made it possible to elucidate the structure of the biopolymer produced. Figure S8 (I) shows the chemical shift of the signals corresponding to different types of carbon atoms in the commercial PHB structure (C = O δ = 169.20 ppm, CH δ = 67.65, CH_2 δ = 40.81 ppm, CH_3 δ = 19.80 ppm), which are compared with the PHA spectrum produced by *B. megaterium* LVN01 is confirmed to be PHB (Figure S8 (II)).

These results are comparable with those reported in other studies for the PHB produced by *B. megaterium* strains (Doi *et al.,* 1986; López *et al.,* 2012; Baikar *et al.,* 2017; Pradhan *et al.,* 2018).

On the other hand, Figure S9 shows the ^{1}H-NMR spectra of the commercial PHB and the PHB obtained from *B. megaterium* LVN01. The signals at δ = 1.30 ppm (c) indicate the presence of the methyl (CH_3) group, δ = 2.50 - 2.64 ppm (b) for the methylene (CH_2) group and δ = 5.28 ppm (a) for the methino group (CH). These values are similar to those reported for the PHB produced by *B. megaterium* strains (Balakrishna Pillai *et al.*, 2016; Baikar *et al.,* 2017; Pradhan *et al.,* 2018).

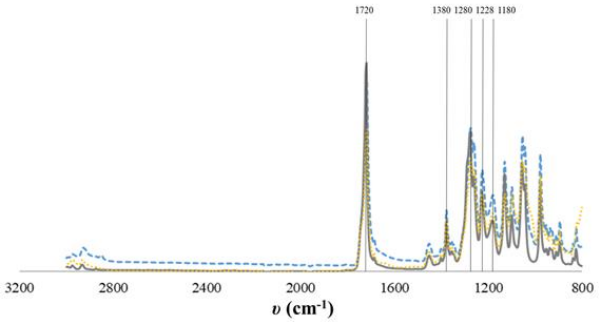

Figure 4. FT-IR spectra for PHB commercial, and PHB produced by *B. megaterium* LVN01 and *B. megaterium BmGD* from glycerol. (—) PHB commercial (Sigma-Aldrich), (·····) PHB from *B. megaterium* LVN01, and (- - -) PHB from *B. megaterium BmGD*.

DISCUSSION

The phylogenetic analyses of 16S rRNA gene-sequence from *B. megaterium* LVN01 showed that this strain belongs to *Bacillus megaterium*. It was confirmed by further analyses based on the comparison of different genes that only own *B. megaterium* strains according to Eppinger and co-workers (Eppinger *et al.,* 2011). For example, genes involved in spore coat forming, gas vesicle biosynthesis as well as the gene responsible for the large size of the cell (*ftsZ*). All of them were detected in *B. megaterium* LVN01 draft genome.

Despite the potentiality of *B. megaterium* for producing PHB from many carbon sources, only a few recombinant clones displaying better performance have been obtained (Hori *et al.,* 2002; Gerber *et al.,* 2015; Grage *et al.,* 2017).

The identification and cloning of the *bm*gd-encoding gene allowed the obtaining of *B. megaterium BmGD*. It is important to highlight that the sequence of *Bm*GD is highly conserved since it has more than 94% of identity with all glycerol dehydrogenases reported in *B. megaterium* strains (Figure S4).

Two different pathways in microorganisms for PHA production from glycerol have been described; an anaerobic process and an aerobic one. Under anaerobic conditions, there are enzymes such as glycerol dehydratase (GDh) (EC 4.2.1.30) and propanal dehydrogenase (PD) (EC 1.2.1.87) that were identified in *Klebsiella* and *Salmonella* strains, respectively (Meng *et al.,* 2014). In contrast, the aerobic process involves NAD^+-dependent glycerol phosphate dehydrogenase (EC 1.1.99.5) and a glycerol kinase (EC 2.7.1.30), and they have been reported in *B. subtilis* (Lindgren and Rutberg, 1974). As was expected, *B. megaterium* lacks glycerol dehydratase. Instead, the strain uptakes glycerol through glycerol dehydrogenase - *Bm*GD (EC 1.1.1.6) and ATP-dependent dihydroxyacetone kinase (EC 2.7.1.29), and finally, it is incorporated in the glycolysis pathway to produce PHA (Figure S1).

Glycerol dehydrogenase is a key enzyme to convert glycerol into PHA by an oxidative pathway. According to the performance of this enzyme, the process to obtain PHA could be successfully achieved. Therefore, the role of this enzyme also could be considered as a bottleneck in this endeavor. This enzyme is an intracellular NAD^+-dependent oxidoreductase present in the glycerol uptake pathway of many microorganisms, and it has been reported as strict dependent on zinc for activity (Ruzheinikov *et al.,* 2001). As was expected, no signal peptide was detected in the sequence, which means that this enzyme is involved in an intracellular catalytic step. Glycerol dehydrogenase catalyzes the oxidation of glycerol to dihydroxyacetone (1,3-dihydroxypropanone) with the reduction of NAD^+ to NADH.

In this study, we evaluated the feasibility to obtain a suitable *B. megaterium* recombinant strain that could overexpress *Bm*GD in order to raise the substrate concentration available inside the cells. Simultaneously, high PHA production was desirable. *Bacillus megaterium BmGD* was able to

grow up using glycerol as the only substrate. It grew up faster than the wild-type under the same operating conditions. *Bacillus megaterium Bm*GD achieved its maximum biomass production at 30 h whereas the wild-type required 48 h. Likewise, it is notorious the improvement of the yield of PHA. Similarly, the recombinant clone contains a higher amount of PHA into the cells in comparison to the wild-type after 30 h of incubation. This performance makes this recombinant microorganism suitable for industrial applications.

A comparison of both strains point out that the wild-type needs 48 h for obtaining the maximum yield and PHB content, and thereafter, it reduces drastically both parameters, whereas the recombinant clone displays improvement sooner, it achieves its maximum performance at 30 h and conserves a high PHB content until 48 h.

Glycerol dehydrogenase and its connection with the PHA metabolism have been studied in detail (Lopar *et al.,* 2014; Meng *et al.,* 2014; Magdouli *et al.,* 2015) Although there are reports of recombinants clones with better glycerol tolerance and improved PHA production (Nikel *et al.,* 2008; Kocharin *et al.,* 2012), up to now, there was no evidence that the overexpression of this enzyme produces recombinant clones with faster growth, higher PHA accumulation as well as higher yield.

A comparison by FTIR and NMR confirmed that both *B. megaterium* strains (wild-type and recombinant) were able to produce PHB from glycerol. The comparison considered commercial PHB, and PHB obtained from the wild-type strain incubated under optimal operating conditions. Further studies will clarify if the optimal operating conditions were altered in the recombinant strain, in terms of biomass and PHA production.

Finally, it is necessary evaluating if further enzymes involve in glycerol uptake in *B. megaterium* can be considered also as a bottleneck. Similarly, a metabolic engineering approach can help us to figure out the real role of glycerol dehydrogenase in PHA biosynthesis. Nevertheless, with the evidence in this study, it is possible to propose that the performance and expression of this enzyme could be considered as a bottleneck in PHA production from glycerol by this strain. It is evident that thanks to this enzyme, it was possible to obtain an engineered strain able to accumulate a higher amount of PHB, faster and with a higher yield.

ACKNOWLEDGMENTS

This study was funded by Departamento Administrativo de Ciencia, Tecnologi□a e Innovacio□n de Colombia (COLCIENCIAS) (Called 647 of 2014). The authors would like to thank COLCIENCIAS for funding. This work is framed at the "Contrato de acceso a recursos genéticos y productos derivados del Ministerio del Medio Ambiente y Desarrollo Sostenible de Colombia Nº95 and Nº159". Special thanks to the Enzymatic Biotechnology (Universidad Complutense de Madrid) and PROBIOM (Universidad Nacional de Colombia) groups for all their support in this work.

REFERENCES

Ashby, R. D., Solaiman, D. K. Y., Strahan, G. D., Levine, A. C., & Nomura, C. T. 2015. Methanol-induced chain termination in poly(3-hydroxybutyrate) biopolymers: Molecular weight control. *International Journal of Biological Macromolecules 74*, 195–201.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., & Zagnitko, O. 2008. The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics 9*(1): 75.

Balakrishna Pillai, A., Jaya Kumar, A., Thulasi, K., & Harikrishnan, K. 2016. Production of short chain length polyhydroxyalkanoates by *Bacillus megaterium* PHB29 from starch feed stock. *International Journal of Current Microbiology and Applied Sciences 5*(7): 816–823.

Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. 2014. Poly-β-hydroxybutyrate production and management of cardboard industry effluent by new *Bacillus* sp. NA10. *Bioresources and Bioprocessing 1*(1): 9.

Campos, M. I., Figueiredo, T. V. B., Sousa, L. S., & Druzian, J. I. 2014. The influence of crude glycerin and nitrogen concentrations on the production of PHA by *Cupriavidus necator* using a response surface methodology and its characterizations. *Industrial Crops and Products 52*: 338–346.

da Silva, G. P., Mack, M., & Contiero, J. 2009. Glycerol: A promising and abundant carbon source for industrial microbiology. *Biotechnology Advances 27*(1): 30–39.

Eppinger, M., Bunk, B., Johns, M. A., Edirisinghe, J. N., Kutumbaka, K. K., Koenig, S. S. K., Creasy, H. H., Rosovitz, M. J., Riley, D. R., Daugherty, S., Martin, M., Elbourne, L. D. H., Paulsen, I., Biedendieck, R., Braun, C., Grayburn, S., Dhingra, S., Lukyanchuk, V., Ball, B., Ul-

Qamar, R., Seibel, J., Bremer, E., Jahn, D., Ravel, J., & Vary, P.S. 2011. Genome sequences of the biotechnologically important *Bacillus megaterium* strains QM B1551 and DSM319. *Journal of Bacteriology 193*(16): 4199–4213.

Grage, K., McDermott, P., & Rehm, B. H. A. 2017. Engineering *Bacillus megaterium* for production of functional intracellular materials. *Microbial Cell Factories 16*(1): 1–12.

Hori, K., Kaneko, M., Tanji, Y., Xing, X. H., & Unno, H. 2002. Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for polyhydroxybutyrate production. *Applied Microbiology and Biotechnology 59*(2–3): 211–216.

Ibrahim, M. H. A. & Steinbüchel, A. 2010. *Zobellella denitrificans* strain MW1, a newly isolated bacterium suitable for poly(3-hydroxybutyrate) production from glycerol. *Journal of Applied Microbiology 108*(1): 214–225.

Jacquel, N., Lo, C. W., Wei, Y. H., Wu, H. S., & Wang, S. S. 2008. Isolation and purification of bacterial poly(3-hydroxyalkanoates). *Biochemical Engineering Journal 39*(1): 15–27.

Johnson, S. L., Daligault, H. E., Davenport, K. W., Jaissle, J., Frey, K. G., Ladner, J. T., Broomall, M., Bishop-Lilly, K. A., Bruce, D. C., Gibbons, H. S., Coyne, S. R., Lo C. C., Meincke, L., Munk, A. C., Koroleva, G. I., Rosenzweig, C. N., Palacios, G. F., Redden, C. L., Minogue, T. D., Chain, P. S. 2015. Complete genome sequences for 35 biothreat assay-relevant *Bacillus* species. *Genome Announcements 3*(2): 10–11.

Kalia, V. C., Prakash, J., & Koul, S. 2016. Biorefinery for glycerol rich biodiesel industry waste. *Indian Journal of Microbiology 56*(2): 113–125.

Kawata, Y. & Aiba, S. 2010. Poly(3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol. *Bioscience, Biotechnology, and Biochemistry 74*(1): 175–177.

Kim, B. S., Lee, S. C., Lee, S. Y., Chang, H. N., Chang, Y. K., & Woo, S. I. 1994. Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. *Biotechnology and Bioengineering*.

Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. 2016. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology 37*: 24–38.

Kocharin, K., Chen, Y., Siewers, V., & Nielsen, J. 2012. Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae*. *AMB Express 2*(1): 52.

Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. 2016. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology 37*: 24–38.

Kumar, P., Patel, S. K. S., Lee, J. K., & Kalia, V. C. 2013. Extending the limits of *Bacillus* for novel biotechnological applications. *Biotechnology Advances 31*(8): 1543–1561.

Lindgren, V. & Rutberg, L. 1974. Glycerol metabolism in *Bacillus subtilis*: gene-enzyme relationships. *Journal of Bacteriology 119*(2): 431–442.

Liu, L., Li, Y., Zhang, J., Zou, W., Zhou, Z., Liu, J., Li, X., Wang, L., & Chen, J. 2011. complete genome sequence of the industrial strain *Bacillus megaterium* WSH-002. *Journal of Bacteriology 193*(22): 6389–6390.

Lopar, M., Špoljarić, I. V., Cepanec, N., Koller, M., Braunegg, G., & Horvat, P. 2014. Study of metabolic network of *Cupriavidus necator* DSM 545 growing on glycerol by applying elementary flux modes and yield space analysis. *Journal of Industrial Microbiology and Biotechnology 41*(6): 913–930.

López, J. A., Naranjo, J. M., Higuita, J. C., Cubitto, M. A., Cardona, C. A., & Villar, M. A. 2012. Biosynthesis of PHB from a new isolated *Bacillus megaterium* strain: Outlook on future developments with endospore forming bacteria. *Biotechnology and Bioprocess Engineering 17*(2): 250–258.

Magdouli, S., Brar, S. K., Blais, J. F., and Tyagi, R. D. 2015. How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production? *Biomass and Bioenergy 74*: 268–279.

Medema, M. H., Blin, K., Cimermancic, P., De Jager, V., Zakrzewski, P., Fischbach, M. A., Weber, T., Takano, E., & Breitling, R. 2011. AntiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Research 39*: 339–346.

Meng, D.-C., Shen, R., Yao, H., Chen, J.-C., Wu, Q., & Chen, G.-Q. 2014. Engineering the diversity of polyesters. *Current Opinion in Biotechnology 29*(1): 24–33.

Mohapatra, S., Maity, S., Dash, H. R., Das, S., Pattnaik, S., Rath, C. C., & Samantaray, D. 2017. *Bacillus* and biopolymer: Prospects and challenges. *Biochemistry and Biophysics Reports 12*: 206–213.

Moreno, P., Yañez, C., Cardozo, N. S. M., Escalante, H., Combariza, M. Y., & Guzman, C. 2015. Influence of nutritional and physicochemical variables on PHB production from raw glycerol obtained from a Colombian biodiesel plant by a wild-type *Bacillus megaterium* strain. *New Biotechnology 32*(6): 682–689.

Możejko-Ciesielska, J. & Kiewisz, R. 2016. Bacterial polyhydroxyalkanoates: Still fabulous? *Microbiological Research 192*(2016): 271–282.

Nikel, P. I., Pettinari, M. J., Galvagno, M. A., & Méndez, B. S. 2008. Poly(3-hydroxybutyrate) synthesis from glycerol by a recombinant *Escherichia coli* arcA mutant in fed-batch microaerobic cultures. *Applied Microbiology and Biotechnology 77*(6): 1337–1343.

Okwuobi, P. N. & Ogunjobi, A. A. 2013. Production and analysis of polyhydroxyalkanoate (PHA) by *Bacillus megaterium* using pure carbon substrates. *World Applied Sciences Journal 28*(9): 1336–1340.

Pappalardo, F., Fragalà, M., Mineo, P. G., Damigella, A., Catara, A. F., Palmeri, R., & Rescifina, A. 2014. Production of filmable medium-chain-length polyhydroxyalkanoates produced from glycerol by *Pseudomonas mediterranea*. *International Journal of Biological Macromolecules 65*: 89–96.

Porras, M. A., Vitale, C., Villar, M. A., & Cubitto, M. A. 2017. Bioconversion of glycerol to poly(HB-co-HV) copolymer in an inexpensive medium by a *Bacillus megaterium* strain isolated from marine sediments. *Journal of Environmental Chemical Engineering 5*(1): 1–9.

Povolo, S., Basaglia, M., Fontana, F., Morelli, A., & Casella, S. 2015. Poly(hydroxyalkanoate) production by *Cupriavidus necator* from fatty waste can be enhanced by *phaZ1* inactivation. *Chemical and Biochemical Engineering Quarterly Journal 29*(2): 67–74.

Povolo, S., Toffano, P., Basaglia, M., & Casella, S. 2010. Polyhydroxyalkanoates production by engineered *Cupriavidus necator* from waste material containing lactose. *Bioresource Technology 101*(20): 7902–7907.

Pradhan, S., Dikshit, P. K., & Moholkar, V. S. 2018. Production, ultrasonic extraction, and characterization of poly (3-hydroxybutyrate) (PHB) using *Bacillus megaterium* and *Cupriavidus necator*. *Polymers for Advanced Technologies 29*(8): 2392–2400.

Reddy, C. S. K., Ghai, R., Rashmi, & Kalia, V. C. 2003. Polyhydroxyalkanoates: An overview. *Bioresource Technology 87*(2): 137–146.

Reddy, S. V., Thirumala, M., & Mahmood, S. K. 2009. A novel *Bacillus* sp. accumulating poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from a single carbon substrate. *Journal of Industrial Microbiology & Biotechnology 36*(6): 837–843.

Rodríguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell-Monfort, M., Braunegg, G., & Marqués-Calvo, M. S. 2015. Influence of glycerol on poly(3-hydroxybutyrate) production by *Cupriavidus necator* and *Burkholderia sacchari*. *Biochemical Engineering Journal 94*: 50–57.

Ruzheinikov, S. N., Burke, J., Sedelnikova, S., Baker, P.J., Taylor, R., Bullough, P. A., Muir, N. M., Gore M. G., & Rice D. W. 2001. Glycerol dehydrogenase: structure, specificity, and mechanism of a family III polyol dehydrogenase. *Structure 9*(9): 789–802.

Sánchez, S. A., Marín, M. A., Mora, A. L., & Yepes, M. del S. 2012. Identification of polyhydroxyalkanoate-producing bacteria in soils contaminated with fique wastes. *Revista Colombiana de Biotecnología 14*(2): 89–100.

Sathiyanarayanan, G., Saibaba, G., Seghal Kiran, G., & Selvin, J. 2013. A statistical approach for optimization of polyhydroxybutyrate production by marine *Bacillus subtilis* MSBN17. *International Journal of Biological Macromolecules 59*: 170–177.

Singh, M., Kumar, P., Ray, S., & Kalia, V. C. 2015. Challenges and opportunities for customizing polyhydroxyalkanoates. *Indian Journal of Microbiology 55*(3): 235–249.

Stewart, I., Schluter, P. J., & Shaw, G. R. 2006. Cyanobacterial lipopolysaccharides and human health - A review. *Environmental Health: A Global Access Science Source 5*: 1–23.

Teeka, J., Imai, T., Reungsang, A., Cheng, X., Yuliani, E., Thiantanankul, J., Poomipuk, N., Yamaguchi, J., Jeenanong, A., Higuchi, T., Yamamoto, K., & Sekine, M. 2012. Characterization of polyhydroxyalkanoates (PHAs) biosynthesis by isolated *Novosphingobium* sp. THA_AIK7 using crude glycerol. *Journal of Industrial Microbiology & Biotechnology 39*(5): 749–758.

Yachdav, G., Kloppmann, E., Kajan, L., Hecht, M., Goldberg, T., Hamp, T., Hönigschmid, P., Schafferhans, A., Roos, M., Bernhofer, M., Richter, L., Ashkenazy, H., Punta, M., Schlessinger, A., Bromberg, Y., Schneider, R., Vriend, G., Sander, C., Ben-Tal, N., & Rost, B. 2014. PredictProtein-an open resource for online prediction of protein structural and functional features. *Nucleic Acids Research 42*: W337-W343.

Zhu, C., Chiu, S., Nakas, J. P., & Nomura, C. T. 2013. Bioplastics from waste glycerol derived from biodiesel industry. *Journal of Applied Polymer Science 130*(1): 1–13.